AD_____

Award Number: DAMD17-00-1-0037

TITLE: Restoring Sensitivity to Apoptosis in Prostate Cancer Cells by Reconstitution of the Tumor Suppressor PTEN

PRINCIPAL INVESTIGATOR: Young E. Whang, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27599-1350

REPORT DATE: January 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2003	Final (1 Jan 00 - 31 Dec 02)

4. TITLE AND SUBTITLE
Restoring Sensitivity to Apoptosis in Prostate Cancer Cells by Reconstitution of the Tumor Suppressor PTEN

5. FUNDING NUMBERS
DAMD17-00-1-0037

6. AUTHOR(S) :
Young E. Whang, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of North Carolina at Chapel Hill
Chapel Hill, North Carolina 27599-1350

E-Mail: ywhang@med.unc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Metastatic prostate cancer almost invariably progresses to the terminal stage despite treatment such as androgen deprivation and chemotherapy and radiation therapy. This resistance to treatment may be due to the resistance to apoptosis in cancer cells. Therefore understanding the molecular basis for resistance to apoptosis is essential for devising novel strategies to sensitize cancer cells to apoptosis. We have been focused on the role of the tumor suppressor PTEN in regulating sensitivity to apoptosis in prostate cancer. We have previously shown that loss of PTEN function leads to excessive antiapoptotic signaling through constitutive activation of the Akt protein kinase. Therefore, we proposed that restoration of PTEN expression may lead to sensitization to apoptotic stimuli such as proapoptotic ligands and chemotherapeutic agents. Over the funding period of this proposal, we have made significant contributions toward substantiating this hypothesis. We have also generated significant data toward the effect of the PTEN signaling pathway in two important transcription factors, NF-κB and the androgen receptor. Our work over the funding period has resulted in 4 published or submitted full-length articles and two abstracts presented at national scientific meetings.

14. SUBJECT TERMS:	15. NUMBER OF PAGES
cell signaling, apoptosis, tumor suppressor	81
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....1

SF 298.....2

Introduction.....4

Body.....4

Key Research Accomplishments.....6

Reportable Outcomes.....6

Conclusions.....7

References.....

Appendices.....9

INTRODUCTION

Metastatic prostate cancer almost invariably progresses to the terminal stage despite treatment such as androgen deprivation and chemotherapy and radiation therapy. This resistance to treatment may be due to the resistance to apoptosis in cancer cells. Therefore understanding the molecular basis for resistance to apoptosis is essential for devising novel strategies to sensitize cancer cells to apoptosis. We have been focused on the role of the tumor suppressor PTEN in regulating sensitivity to apoptosis in prostate cancer. We have previously shown that the majority of advanced prostate cancer tumors has lost PTEN function and that loss of PTEN function leads to excessive antiapoptotic signaling through constitutive activation of the Akt protein kinase. Therefore, we proposed that restoration of PTEN expression may lead to sensitization to apoptotic stimuli. Over the funding period of this proposal, we have made significant contributions toward substantiating this hypothesis. We have also generated significant data toward the effect of the PTEN signaling pathway on two important antiapoptotic transcription factors, NF-κB and the androgen receptor. Our work over the funding period has resulted in 4 published or submitted full-length articles in peer-reviewed journals and two abstracts presented at national scientific meetings.

BODY

Statement of Work Task 1: To characterize the role of PTEN in the regulation of anoikis

Statement of Work Task 2: To characterize the role of the effect of PTEN expression in sensitizing cells to apoptotic stimuli

Annual reports covering the periods of Jan.-Dec. 2000 and Jan.-Dec. 2001 detail the data addressing statement of work task 1 and 2. We have completed the proposed experiments and data have been published in the article provided in the appendix (Yuan, X.J. and Whang, Y. (2002) PTEN sensitizes prostate cancer cells to death receptor-mediated and drug-induced apoptosis through a FADD-dependent pathway. Oncogene, 21, 319-327.)

Statement of Work Task 3: To characterize the in vivo effect of PTEN expression on tumorigenicity and sensitivity to chemotherapy
3A: determine the tumorigenicity of PTEN expressing xenograft cells in mice.
3B: determine the effect of direct injection of PTEN adenovirus on pre-formed tumors.
3C: determine the effect of combining injection of PTEN adenovirus and chemotherapy.

For task 3A, we asked if PTEN expression through adenovirus mediated gene transduction will lead to loss of tumorigenicity of CWR22 prostate xenograft cells. CWR22 xenograft cells were freshly harvested and infected with PTEN adenovirus ex

vivo and the implanted back into immunodeficient mice. As shown in Figure 1, PTEN expression completely inhibited the ability of CWR22 xenograft cells to form tumor.

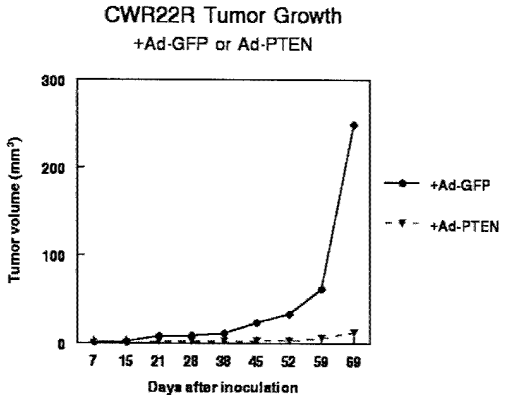


Fig. 1. Adenovirus-mediated PTEN expression completely suppresses the ability of CWR22 prostate xenograft cells to form tumor in nude mice. Freshly harvested CWR22 cells were incubated with Ad-PTEN or Ad-GFP at moi of 50. Then 10^6 cells were inoculated per site. The tumor volume shown is the mean of 6 tumor sites. In addition to data shown, another independent experiment shows similar results.

Task 3B or 3C involves direct intratumoral injection of PTEN adenovirus on pre-formed prostate tumors. However, after extensive discussions with other investigators who have attempted this approach of gene transduction, we felt that this approach is unlikely to result in successful PTEN transduction in the majority of tumor cells. The PTEN adenovirus appears to lower the threshold to apoptosis, but does not potently induce apoptosis by itself and the direct injection of PTEN adenovirus in the tumors will only affect cells in the needle track. It is unlikely that the direct injection of the PTEN adenovirus will have any discernible effect on established tumors. Therefore, we did not pursue experiments in these tasks.

Additional work accomplished

We pursued experiments focusing on the effect of PTEN expression on the transcriptional activity of transcription factors such as NF-κB and the androgen receptor. We've made significant progress in investigating the effect of PTEN on these transcription factors with antiapoptotic functions and we've been able to show that PTEN inhibits the transcriptional function of these proteins. These demonstrate the additional mechanisms by which loss of PTEN leads to resistance to apoptosis. Data from this line of investigation have been published in Mayo, M., Madrid, L., Westerheide, S., Jones, D., Yuan, X., Baldwin, A., and Whang, Y. (2002) PTEN blocks tumor necrosis factor-induced NF-κB-dependent transcription by inhibiting the transactivation potential of the p65 subunit. J. Biol. Chem., 277, 11116-11125, and this article is appended. Also, a manuscript by Nan, B., Snabboon, T., Unni, E., Yuan, X., Whang, Y., and Marcelli, M. (2003) The PTEN tumor suppressor is a negative modulator of androgen receptor transcriptional activity, has been submitted for publication to Journal of Molecular Endocrinology and is provided in the appendix.

KEY RESEARCH ACCOMPLISHMENTS

We have made the following conclusions from our research:

- Adenovirus-mediated PTEN expression suppresses constitutive Akt phosphorylation in prostate cancer cells.
- PTEN sensitizes prostate cancer cells to apoptosis induced by several different classes of chemotherapeutic agents.
- PTEN sensitizes prostate cancer cells to apoptosis induced by several different death ligands, such as tumor necrosis factor-α, anti-Fas antibody, and TRAIL.
- PTEN-mediated apoptosis is accompanied by caspase activation and is inhibited by caspase inhibitor z-VAD-fmk.
- PTEN-mediated apoptosis is dependent on lipid phosphatase activity.
- PTEN-mediated apoptosis involves a FADD-dependent pathway.
- PTEN-mediated apoptosis proceeds through BID cleavage.
- Bcl-2 blocks PTEN-mediated apoptosis.
- Adenovirus-mediated PTEN expression inhibits the tumorigenicity of CWR22 prostate cancer xenograft cells.
- PTEN blocks tumor necrosis factor-induced NF-κB-dependent transcription by inhibiting the transactivation potential of the p65 subunit.
- PTEN suppresses the transcriptional activity of the androgen receptor in prostate cancer cells.

REPORTABLE OUTCOMES

1) Abstract presented as a poster at the 92th Annual Meeting of the American Association for Cancer Research in March, 2001, New Orleans, Louisiana.
Yuan, X., Ji, X., and Whang, Y. (2001) The PTEN Tumor Suppressor Sensitizes Prostate Cancer Cells to Apoptosis through a FADD-dependent Pathway.

2) Abstract presented as a poster at the 93rd Annual Meeting of the American Association for Cancer Research in April, 2002, San Francisco, California.
Nan, B., Whang, Y., and Marcelli, M. (2002) Interactions between the androgen receptor (AR) and PTEN signaling pathways in AR(+) and PTEN(+) or (-) prostate cancer cell lines.

3) Articles published in peer reviewed journals:

Gupta, S., Suffrein, S., Plattner, R., Tencati, M., Gray, C., Whang, Y., and Stanbridge, E. (2001) Role of phosphoinoside 3-kinase in the aggressive tumor growth of HT1080 human fibrosarcoma cells. Mol. Cell. Biol., 21, 5846-5856.

Yuan, X.J. and Whang, Y. (2002) PTEN sensitizes prostate cancer cells to death receptor-mediated and drug-induced apoptosis through a FADD-dependent pathway. Oncogene, 21, 319-327.

Mayo, M., Madrid, L., Westerheide, S., Jones, D., Yuan, X., Baldwin, A., and Whang, Y. (2002) PTEN blocks tumor necrosis factor-induced NF-κB-dependent transcription by inhibiting the transactivation potential of the p65 subunit. J. Biol. Chem., 277, 11116-11125.

4) Articles submitted for publication:

Nan, B., Snabboon, T., Unni, E., Yuan, X., Whang, Y., and Marcelli, M. (2003) The PTEN tumor suppressor is a negative modulator of androgen receptor transcriptional activity, manuscript submitted to J. Mol. Endocrin.

BIBLIOGRAPHY

Gupta, S., Suffrein, S., Plattner, R., Tencati, M., Gray, C., **Whang, Y.,** and Stanbridge, E. (2001) Role of phosphoinoside 3-kinase in the aggressive tumor growth of HT1080 human fibrosarcoma cells. Mol. Cell. Biol., 21, 5846-5856.

Yuan, X.J. and **Whang, Y.** (2002) PTEN sensitizes prostate cancer cells to death receptor-mediated and drug-induced apoptosis through a FADD-dependent pathway. Oncogene, 21, 319-327.

Mayo, M., Madrid, L., Westerheide, S., Jones, D., Yuan, X., Baldwin, A., and **Whang, Y.** (2002) PTEN blocks tumor necrosis factor-induced NF-κB-dependent transcription by inhibiting the transactivation potential of the p65 subunit. J. Biol. Chem., 277, 11116-11125.

Nan, B., Snabboon, T., Unni, E., Yuan, X., **Whang, Y.,** and Marcelli, M. (2003) The PTEN tumor suppressor is a negative modulator of androgen receptor transcriptional activity, manuscript submitted to J. Mol. Endocrin.

Yuan, X., Ji, X., and **Whang, Y.** (2001) The PTEN tumor suppressor sensitizes prostate cancer cells to apoptosis through a FADD-dependent pathway. Proceedings of the American Association for Cancer Research 42, 1311.

Nan, B., **Whang, Y.,** and Marcelli, M. (2002) Interactions between the androgen receptor (AR) and PTEN signaling pathways in AR(+) and PTEN(+) or (-) prostate cancer cell lines. Proceedings of the American Association for Cancer Research 43, 2512.

CONCLUSIONS

Resistance to apoptosis is a significant problem that directly contributes to the morbidity and mortality associated with prostate cancer. We have focused on PTEN as an important regulator of apoptosis in prostate cancer cells and have hypothesized that if

loss of PTEN leads to excessive antiapoptotic signaling through constitutive activation of Akt kinase, restoration of PTEN expression may lead to sensitization to apoptotic stimuli. We have generatde a significant amount of data in support of this hypothesis. We have shown that PTEN sensitizes prostate cancer cells to apoptosis induced by several different classes of chemotherapeutic agents and by death ligands and that PTEN-mediated apoptosis is accompanied by caspase activation. Furthermore, we have also demonstrated that PTEN-mediated apoptosis involves a FADD-dependent pathway and that PTEN-mediated apoptosis proceeds through BID cleavage. In addition, we have made an important observation that the PTEN signaling pathway negatively modulate the activity of two transcription factors critically involved in prostate cancer pathogenesis, namely NF-κB and the androgen receptor.

These findings are important because they delineate the functional consequences of loss of PTEN function, a common genetic abnormality in prostate cancer, and show how loss of PTEN leads to excessive antiapoptotic signaling. Our work has produced two abstracts reported at national meetings, three published articles, and one manuscript submitted for publication. Better understanding of apoptotic signaling in prostate cancer is likely to be helpful in designing targeted approaches to prostate cancer therapy and we believe that our work has made a significant contribution in this field.

List of Personnel Supported by this Project

Young E. Whang, Principal Investigator
Xiu-Juan Yuan, Research Associate

Appendices

Item #1: Abstract presented as a poster at the 92th Annual Meeting of the American Association for Cancer Research in March, 2001, New Orleans, Louisiana
Yuan, X., Ji, X., and Whang, Y. (2001) The PTEN Tumor Suppressor Sensitizes Prostate Cancer Cells to Apoptosis through a FADD-dependent Pathway.

Item #2: Abstracted presented as a poster at the 93rd Annual Meeting of the American Association for Cancer Research in April, 2002, San Francisco, California.
Nan, B., Whang, Y., and Marcelli, M. (2002) Interactions between the androgen receptor (AR) and PTEN signaling pathways in AR(+) and PTEN(+) or (-) prostate cancer cell lines.

Item #3: Published Article
Gupta, S., Suffrein, S., Plattner, R., Tencati, M., Gray, C., Whang, Y., and Stanbridge, E. (2001) Role of phosphoinoside 3-kinase in the aggressive tumor growth of HT1080 human fibrosarcoma cells. Mol. Cell. Biol., 21, 5846-5856.

Item #4: Published Article
Yuan, X.J. and Whang, Y. (2002) PTEN sensitizes prostate cancer cells to death receptor-mediated and drug-induced apoptosis through a FADD-dependent pathway. Oncogene, 21, 319-327.

Item #5: Published Article
Mayo, M., Madrid, L., Westerheide, S., Jones, D., Yuan, X., Baldwin, A., and Whang, Y. (2002) PTEN blocks tumor necrosis factor-induced NF-κB-dependent transcription by inhibiting the transactivation potential of the p65 subunit. J. Biol. Chem., 277, 11116-11125.

Item #6: Articles submitted for publication:
Nan, B., Snabboon, T., Unni, E., Yuan, X., Whang, Y., and Marcelli, M. (2003) The PTEN tumor suppressor is a negative modulator of androgen receptor transcriptional activity, manuscript submitted to J. Mol. Endocrin.

#1311 The PTEN Tumor Suppressor Sensitizes Prostate Cancer Cells to Apoptosis through a FADD-Dependent Pathway. Xiu-Juan Yuan, Xiang Ji, and Young E. Whang. *University of North Carolina, Chapel Hill, NC.*

PTEN is a recently identified tumor suppressor gene on chromosome 10q23 frequently inactivated in a wide range of human tumors including advanced prostate cancer. PTEN is a phosphatase that dephosphorylates the lipid second messenger phosphatidylinositol (PI)-3-phosphate and reverses the action of PI 3-kinase. Loss of PTEN function leads to constitutive activation of downstream mediators of PI 3-kinase, including Akt/protein kinase B. Since Akt/protein kinase B has been implicated in antiapoptotic signaling by inactivating several key proapoptotic proteins, loss of PTEN function may promote carcinogenesis by conferring tumor cells resistance to multiple apoptotic stimuli, including death ligands and chemotherapeutic agents. In this study, we test the hypothesis that reconstitution of PTEN expression will sensitize tumor cells to various apoptotic stimuli and investigate the signaling pathway utilized by PTEN to sensitize cells to apoptosis. We used adenovirus-mediated PTEN expression to reconstitute PTEN in PTEN- null, androgen-dependent LNCaP prostate cancer cells. This leads to suppression of constitutive activation of Akt. We examined the effect of PTEN on sensitivity to apoptosis. At 48 hours after infection, PTEN expression by itself did not induce apoptosis. However, it markedly sensitized LNCaP cells to apoptosis induced by death receptor signaling, such as tumor necrosis factor and agonistic anti-Fas monoclonal antibody. In addition, PTEN expression also sensitized cells to non-death receptor mediated apoptosis, such as staurosporine and etoposide. Apoptosis induced by PTEN expression and another apoptotic stimulus involved caspase activation, as shown by increase in DEVD-peptide specific caspase-3 activity and inhibition of apoptosis by z-VAD-fmk caspase inhibitor. Lipid phosphatase activity of PTEN was absolutely required for apoptosis since the PTEN G129E mutant defective in lipid phosphatase but retaining tyrosine phosphatase was unable to sensitize cells to apoptosis. Apoptosis induced by PTEN expression and another apoptotic stimulus was completely blocked by overexpression of Bcl-2, implicating the involvement of the mitochondrial pathway. To define the requirement for components of death receptor signaling, we co-expressed dominant negative FADD (Fas associated death domain) by adenovirus. DN FADD blocked apoptosis induced by PTEN and TNF or Fas. Surprisingly, DN FADD also blocked apoptosis induced by PTEN and staurosporine or staurosporine alone. Apoptosis induced by staurosporine could not be blocked by neutralizing monoclonal antibodies against death receptors Fas and TNFR1. These data suggest that both death receptor-mediated and chemical-induced apoptosis requires FADD-dependent signaling and that PTEN sensitizes cells to apoptosis through a FADD-dependent signaling pathway.

#1312 Sensitization to Proapoptotic Agents by Ectopic Akt1 Expression in Prostate Cancer Cells. Hamid Boulares, Zohra El Mkami, Robert Glazer, and Shakeel Ahmad. *Department of Biochemistry, Georgetown Univ. Medical Center, Washington, DC, Department of Oncology, Lombardi Cancer Center, Georgetown Univ. Medical Center, Washington, DC, and Department of Pharmacology, Lombardi Cancer Center, Georgetown Univ. Medical Center, Washington, DC.*

The protein-serine/threonine kinase, Akt/PKB is the cellular homologue of the retroviral oncogene v-akt, and is one of the immediate downstream effector of phosphatidylinositol 3-kinase (PI 3-kinase). The major mechanism by which growth factors receptors promote cell survival is through the pathway leading to the activation of PI 3-kinase and Akt. Akt is also widely known to induce cell survival in a number of cell types by intervening with the apoptotic cascade triggered by different apoptotic stimuli. In the present study, we report that while overexpression of Akt enhances cell proliferation in TSU prostate cancer cells, it also sensitizes the cells to apoptosis rather than conferring protection when challenged with the proapoptotic agents, staurosporine, TNF or FasL. The degree of sensitization varies with the different apoptotic agents with staurosporine being the most effective. To delineate the cause of enhanced apoptosis in Akt over-expressing cells, the effect of staurosporine treatment and other apoptosis inducing agents on cytochrome c release, activation of caspases-3 and -9, PARP cleavage, and DNA breakage into 50 kb fragments were measured. All parameters were greatly enhanced by the three proapoptotic agents in Akt-expressing cells. These results suggest that ectopically expressed Akt itself might be affected by staurosporine to interfere with the early onset of a survival pathway in prostate cancer cells. Indeed, staurosporine was found to induce the proteolysis of Akt to a 40-45 kD fragment. Studies are underway to characterize the proteolytic Akt fragment for kinase activity as well as the downstream effect of the fragment on the phosphorylation status of proapoptotic proteins in these cells.

#1313 Cdc25a Phosphatase Interacts with Akt and Suppresses Apoptosis Induced by Serum Deprivation. Georg Krupitza, Christina Leisser, Gerhard Fuhrmann, Georg Rosenberger, Michael Grusch, Thomas Halama, Isabella Mosberger, Christa Cerni, and Thomas Szekeres. *University of Vienna, Vienna, Austria.*

The phosphatase Cdc25A was shown to be a target of the transcription factor c-Myc. Myc-induced apoptosis appeared dependent on Cdc25A expression and Cdc25A over-expression could substitute for Myc-triggered apoptosis. These findings suggested that an important downstream component of Myc-mediated apoptosis was identified. However and in contrast, we recently reported that during TNFa-induced apoptosis, which required c-Myc function, Cdc25A was down-regulated in a human carcinoma cell line. We now provide evidence that Cdc25A rendered the non-transformed rat embryonic cell line 423 refractory to apoptosis, which was induced by serum deprivation and in absence of detectable c-myc levels. The survival promoting activity of cdc25A was abolished upon infection of cells with a full length cdc25A antisense construct. To identify the signalling proteins mediating the survival function of the phosphatase, cdc25A- and akt- over-expressing pooled clones were exposed to selected chemicals, which inhibit or activate key proteins in signalling pathways. Inhibition of apoptosis by SU4984, NF023 and Rapamycin placed Cdc25A and Akt function downstream of FGF.R, PDGF.R, and compensated G-protein- and PP2A- activity. Interestingly, upon treatment with LY-294002, cdc25A- and akt- over-expressing clones exhibited similar apoptotic patterns as control cells, which indicates that neither Akt- nor Cdc25A- mediated survival functions are dependent on PI.3 kinase activity in rat 423 cells. In cdc25A-overexpressing cells increased levels of serine 473 phosphorylated Akt were found, which co-precipitated with Cdc25A and Raf1. Since activation of proteins requires dephosphorylation of particular residues in addition to side-specific phosphorylation, the anti-apoptotic effect of Cdc25A might derive from its participation in a ternary protein complex with phosphoAkt and Raf1, two prominent components of survival pathways.

#1314 The Multi-Substrate Adapter Gab1 Regulates Hepatocyte Growth Factor/Scatter Factor (HGF/SF)-C-Met Signaling for Cell Survival and DNA Repair. S. Fan, YX Ma, M. Gao, RQ Yuan, Q. Meng, I.D. Goldberg, M. Park, and E. M. Rosen. *Long Island Jewish Medical Center, New Hyde Park, NY.*

Hepatocyte growth factor/scatter factor (HGF/SF) is a mediator of epithelial cell motility, morphogenesis, angiogenesis, and tumorigenesis. HGF/SF protects cells against DNA damage by a pathway from its receptor c-Met→phosphatidylinositol-3kinase [PI3K]→c-Akt, resulting in enhanced DNA repair and decreased apoptosis. We now show that protection against the DNA-damaging agent adriamycin [ADR] (a topoisomerase IIα inhibitor) requires the Grb2 binding site of c-Met; and over-expression of the Grb2-associated binder Gab1 - a multi-substrate adapter required for epithelial morphogenesis - inhibits the ability of HGF/SF to protect MDCK epithelial cells against ADR. In contrast to Gab1 and its homolog Gab2, over-expression of c-Cb1, another multisubstrate adapter that associates with c-Met did not affect protection. The ability of Gab1 to block HGF/SF-mediated activation of c-Akt and protection did not require its pleckstrin homology or SHP2 phosphatase-binding domains but did require the PI3K-binding domain. HGF/SF protection of parental MDCK cells was blocked by wortmannin, expression of PTEN, and dominant negative mutants of Akt and Pak1; and the protection of cells over-expressing Gab1 was restored by wild-type or activated mutants of Akt and Pak1, but not by the PI3K catalytic submit. These findings suggest that the adapter Gab1 "re-directs" c-Met signaling through PI3K away from a c-Akt/Pak1 cell survival pathway.

#1315 EGFR Induced Activation of NF-κB in Mesothelial Cells by Asbestos Is Important in Cell Survival. Stephen P. Faux, Catherine Houghton, William Swain, John G. Edwards, Ricky Sharma, Simon Plummer, and Ken J. O'Byrne. *Department of Oncology, University of Leicester, Leicester, UK, and MRC Toxicology Unit, University of Leicester, Leicester, UK.*

Over-expression of the epidermal growth factor receptor (EGFR) is a common finding in many solid tumors, including lung, breast and mesothelioma, and has been shown to correlate with both a poor prognosis and resistance to radiation and chemotherapy. The proliferative response and resistance afforded by EGFR activation in these tumors may allow for the rapid outgrowth of drug resistant cells. Recent evidence suggests that up-regulation and activation of EGFR may play a critical role in early carcinogenic events. Although toxic to a proportion of MET 5A cells, carcinogenic asbestos fibers upregulate the expression of the EGFR. Exposure of MET 5A cells to asbestos leads to the activation of nuclear factor-κB (NF-κB), a transcription factor important in the regulation of a number of genes intrinsic to inflammation, proliferation and lung defences. This study set out to examine the relationship between EGFR and NF-κB in MET 5A cells exposed to asbestos fibers and the impact of NF-κB activation on MET 5A cell survival. Using gel mobility shift assays we have shown that crocidolite asbestos increased the DNA binding activity of NF-κB in MET 5A cells. Pre-treatment with (a) curcumin, a potent antioxidant non-selective inhibitor of NF-κB derived from the spice turmeric, or (b) the selective EGFR tyrosine kinase inhibitor, PKI166 (Novartis), inhibited the DNA binding of NF-κB mediated by crocidolite asbestos fibers. In addition, crocidolite in combination with either curcumin or PKI166 increased the cellular toxicity of crocidolite compared to that observed with crocidolite alone. Finally, an NF-κB "decoy" peptide inhibited crocidolite induced MET 5A cell proliferation. Our results would suggest that the induction of NF-κB by crocidolite asbestos in MET 5A cells is via a signalling pathway linked to EGFR. This survival signal protects MET 5A cells from the cellular toxicity of crocidolite. Modulation of the asbestos-mediated EGFR/NF-κB signalling pathway may be important in the development of novel therapeutic strategies for both the chemoprevention and treatment of malignant mesothelioma.

#1316 Protein Kinase CK2 Function in Blocking Drug-Induced Apoptosis in Cancer Cells. S. Yu, H. Wang, A. Davis, and K. Ahmed. *V.A. Medical Center and University of Minnesota, Minneapolis, MN.*

Protein kinase CK2 (formerly casein kinase II) is well-known to be associated with cell growth and proliferation, and has been found to be elevated in tumor cells. Nuclear matrix (NM) is a major site of CK2 signaling for these actions. Here we report that NM-associated CK2 also plays an additional role as providing protection against drug-mediated apoptosis in cancer and other cells. We examined the response of nuclear CK2 to induction of apoptosis in prostate cancer cells through chemical agents such as etoposide (Et) and diethylstilbestrol (DES).

#2509 Resveratrol acts as an agonist in breast cancer cells transfected with ERα. Anait S. Levenson, Jun Horiguchi, Laura A. Simons, James E. Ward III, Katherine M. Pease, and V. Craig Jordan. *Northwestern University Medical School, Chicago, IL.*

Resveratrol (Res) (3,5,4'- trihydroxy-trans-stilbene) is a phytoestrogen found in grapes and present in red wine. Based on epidemiological studies Res may act as a cancer chemopreventive compound. The inhibitory effects on breast cancer cell proliferation *in vitro* by Res can be explained, partially, by its interaction with the estrogen receptor (ER). Moreover, Res differentially affects the transcriptional activity of ERα and ERβ in an ERE sequence-dependent manner. The aim of our work was to study the effects of Res at both the cellular (cell growth) and the molecular (TGFα gene activation) levels in breast cancer cells stably transfected with wtERα (D351) and mutant ERα (D351Y). Results show that Res had inhibitory effects on the growth of these cells in a dose-dependent fashion, like estradiol (E2). Interestingly, while pure antiestrogen ICI 182,780 (ICI) was able to block the inhibitory effects of E2 on cell proliferation, it was not able to block the inhibitory effects of Res suggesting that antiproliferative effects of Res are not fully ER-mediated. In agreement with this observation Res decreased cell proliferation of parental MDA-MB-231 cells. We used activation of TGFα mRNA detected by Northern blot as a marker of E2 responsiveness. We found a dose-dependent (10^{-8}-10^{-4}M) induction of TGFα mRNA by Res, indicating that Res acts as an estrogen agonist in both cell lines. ICI was able to block Res-induced activation of TGFα implicating ER-mediated events. Res maximally stimulated the expression of TGFα mRNA at a concentration of $5x10^{-5}$M, to the about the same extent as 10^{-9}M E2. However, when Res and E2 were both present, there was no further stimulation of TGFα mRNA. Further studies with both cell lines demonstrated that Res acted as an agonist in a dose-dependent manner in the presence of low concentrations of E2 (10^{-10}-10^{-11}M) and did not act as an agonist in the presence of high (10^{-9}-10^{-8}M) concentrations of E2. To determine whether Res belongs to class I or class II estrogens (*Jordan et al, Cancer Res, 61, 6619-23, 2001*) we examined Res with D351G ERα in the TGFα assay. As was predicted based on the structure of Res (planar and related to E2 and DES), it was an estrogen agonist with D351G ERα and this agonistic action was blocked by array of SERMs and ICI. Therefore, Res belongs to the class I estrogens and produces its estrogen-like action through the AF2 coactivator binding site in synergy with AF1. Continued analysis of ER-Res interaction on estrogen-regulated genes and coregulator proteins is necessary to better understand the beneficial anticancer, cardioprotective and antiosteoporotic effects of Res mediated by the ER. Supported by SPORE # CA89018-01, IDPH Penny Severns Breast and Cervical Cancer Research Fund (ASL) and the Avon Products Foundation.

#2510 Development of a two-stage cysteine (Cys) alkylation procedure to accurately evaluate the functional effect of oxidative stress on the estrogen receptor's DNA-binding domain (ER-DBD). Jose E. Meza, Gary Scott, Alma Burlingame, Christopher Benz, and Michael Baldwin. *University of California, San Francisco, San Francisco, CA, and Buck Institute for Age Research, Novato, CA.*

Antiestrogen therapy is the treatment of choice for most patients with estrogen receptor (ER)-positive breast tumors. However, it is unclear why nearly 50% of ER-positive tumors demonstrate clinical resistance to this form of ER-targeted therapy. Interestingly, nearly a third of primary ER-positive breast tumors contain immunoreactive ER that is unable to bind *in vitro* to a probe containing its cognate DNA estrogen response element (ERE). For some of these tumors, thiol reduction restores this DNA-binding function, suggesting that loss of ER DNA-binding may be due in part to *in vivo* oxidation of Cys residues within the ER DNA-binding domain (ER-DBD). Mass spectrometric analysis of recombinant ER-DBD determined that this defect is indeed due to oxidation of the two zinc fingers within this domain, with zinc finger two appearing to be the most susceptible to oxidation. In this study, we focus on the development of a protocol that will allow us to accurately study the functional effect of oxidative stress on the ER-DBD in the context of the full-length (67 kDa) ER protein. The procedure involves the initial selective carboxymethylation of non-oxidized Cys residues in ER with iodoacetic acid, an in-gel reduction and a second alkylation step with doubly labeled ^{13}C bromoacetic acid to identify Cys residues that were originally oxidized, followed by in-gel proteolytic digestion and peptide analysis by liquid chromatography / mass spectrometry (LC/MS). Employing this two-stage alkylation procedure has allowed us to 1) circumvent *ex-vivo* oxidation of Cys residues within the ER-DBD, which readily occurs via thiol-disulphide exchange reactions, 2) quantitatively assess the redox state of each Cys within the ER-DBD after treatment of the full-length protein with various concentrations of oxidizing agents (i.e. hydrogen peroxide and diamide) and 3) probe the structure of the zinc fingers through their reactivity to the alkylating agents. Additionally, we are in the process of using the protocol to analyze *in vivo*-induced Cys oxidation in ER purified from cultured breast cancer cells to correlate these oxidant stress-induced structural changes with the measurable loss of ER DNA-binding function. The ultimate goal of these studies is to develop a clinical test that will identify breast tumors possessing oxidatively damaged ER that are less likely to respond to ER-targeted endocrine therapy.

#2511 Retinoic acid activates the small GTPases Rac1 and Cdc42 through c-Src kinase in differentiating human neuroblastoma cells. Nandini Dey, Pradip De, Donald Durden, and Kent Robertson. *Indiana University, Wells Center for Pediatric Research, Indianapolis, IN.*

Retinoic acid (RA) induces neuronal differentiation of neuroblastoma (NB) cells with neurite outgrowth and growth inhibition, however downstream signaling pathways of RA receptor activation leading to neurite development are poorly characterized. LAN5 human NB cells exhibit growth inhibition, induced expression of $p27^{kip1}$ and extensive arborization of neurites in response to RA for 8-10 days. Pull down immunoblots were evaluated for activated Rac1 (GTP bound Rac1) and Cdc42 from LAN5 cells treated with 10^{-5} M RA for 24, 48, 72 hours and 7 days. The activation of Rac1 and Cdc42 appeared after 24 hours and peaked at 48 hours. Since we prevoiusly observed that Src is essential to RA induced NB differentiation, we also examined pull down immunoblots of activated Rac1 and Cdc42 both following exposure to the Src inhibitor, PP1, and with retroviral (LXSN vector) over-expression of Csk, the physiological inhibitor of Src. RA-induced time dependent activation of Rac1 and Cdc42 was blocked by both pre-treatment with PP1 and with Csk over-expression in LAN5 cells suggesting that RA-induced small GTPase activation is mediated through Src. These observations correlated with a block in morphologic RA-induced neuronal differantiation of LAN5 cells by both PP1 and high level expression of Csk. The conclusion from these studies is that RA-induced neuronal differntiation of NB cells as well as activation of Rho small GTPases appears to mediated through Src tyrosine kinase.

#2512 Interactions between the androgen receptor (AR) and PTEN signaling pathways in AR(+) and PTEN(+) or (-) prostate cancer cell lines. Bicheng Nan, Young E. Whang, and Marco Marcelli. *Baylor College of Medicine/ VA Medical Center, Houston, TX, and University of North Carolina School of Medicine, Chapel Hill, NC.*

Introduction The mechanism leading to androgen-independent prostate cancer is poorly understood. Many prostate cancers are associated with loss of PTEN. This tumor suppressor gene works by antagonising the PI3K pathway to induce apoptosis and growth arrest. In the prostate, AR is believed to work by antagonizing apoptosis and inducing cell proliferation. Because this two molecules play opposing roles in prostatic epithelium, and they have previously been shown to antagonize each other, we performed a number of experiments using PTEN positive (+) or negative (-) prostate cancer cell lines to further characterize the degree of AR-PTEN interaction. Methods PTEN (-) LNCaP and PTEN (+) LAPC4 prostate cancer cells were used. Both these cell lines are androgen receptor (AR) positive (+). Cells treated with or without 2 nM dihydrote stosterone (DHT) were infected with various combinations of adenovirus ARR2PB-Bax, which contains a Bax cDNA linked to the AR-dependent and prostate specific ARR2PB promoter, and adenovirus CMV-PTEN, which contains a PTEN cDNA under the control of the CMV promoter. At the end of the experiments cells were analyzed for the expression of Bax (an index of an exogenous DHT-stimulated promoter), and PSA (an index of an endogenous DHT-stimulated promoter). Results In LNCaP cells re-expression of PTEN was associated with decreased expression of both the endogenous (PSA) and exogenous (Bax) AR-dependent molecules. Similarly, overexpression of PTEN in LAPC4 cells inhibited AR-induce d Bax and PSA expression. Conclusions: These data suggest that PTEN antagonizes the AR signaling pathway. This effect is not cell line or promoter-dependent. As PTEN is frequently inactivated in androgen independent prostate cancer, these results sug gest that loss of PTEN function may facilitate activation of AR signaling and progression to androgen independence.

#2513 Regulation of ERb-mediate transcription by AKT and MAPK is mediated through AF-2 recruitment of p160 coactivators. Bich N. Duong, Steven Elliott, Lilia Melnik, Barbara Beckman, Jawed Alam, John McLachlan, Matthew Burow, and Yan Tang. *Tulane University, New Orleans, LA.*

Regulation of estrogen receptor activation by growth factors occurs through both direct phosphorylation of the receptor and activation of receptor interaction coactivator proteins. Here we investigate the roles of insulin-like growth factor-1 (IGF-1)-mediated signaling cascades on the regulation of estrogen receptor activity. We demonstrate that constitutive active mutants of AKT (CA-AKT) and MKK1 (CA-MKK1) can greatly potentiate both ERa and ERb activity. Our results also indicate that AKT potentiation of ERb activity occurs predominately through the AF-2 domain. Given the role of coactivators in regulation of AF-2 functions of both ERa and b, we further investigate the possible involvement of SRC-1 and GRIP in AKT and ERb-AF-2 crosstalk by using the mammalian two hybrid system. We consequently demonstrate that AKT can increase recruitment of SRC-1 and GRIP to the ERb-AF2 domain, while MKK1 can enhance GRIP recruitment to the same domain. In summary, these data suggest a novel mechanism by which IGF-1 activation of the AKT and MKK1 signaling cascades can regulate ERb activity through affecting p160 coactivator recruitment to the AF-2 domain.

#2514 Inhibition of human pancreatic cancer cell growth and mitogenic signaling by insulin receptor substrate-1 pleckstrin homology domains. Marko Kornmann, Hariolf Fackler, Pierluici Di Sebastiano, Murray Korc, and Marco Falasca. *University of Ulm, Ulm, Germany, University of California, Irvine, CA, and The Rayne Institute University College, London, UK.*

Insulin receptor substrate-1 (IRS-1) mediates mitogenic insulin-like growth factor (IGF-I) and insulin signaling. Insulin, IGF-I and its cell surface tyrosine kinase receptor IGF-IR are co-expressed at high levels in many human pancreatic cancers. We have previously demonstrated that IRS-1 is present at high levels in pancreatic cancer cells. The aim of this study was to investigate the effect of inhibition of IRS-1 signaling on cell growth and activation of mitogen-activated

MOLECULAR AND CELLULAR BIOLOGY, Sept. 2001, p. 5846–5856
0270-7306/01/$04.00+0 DOI: 10.1128/MCB.21.17.5846–5856.2001

Vol. 21, No. 17

Role of Phosphoinositide 3-Kinase in the Aggressive Tumor Growth of HT1080 Human Fibrosarcoma Cells

SWATI GUPTA,[1] SELMA STUFFREIN,[1] RINA PLATTNER,[1]† MICHAEL TENCATI,[1] CHRISTA GRAY,[1] YOUNG E. WHANG,[2] AND ERIC J. STANBRIDGE[1]*

Department of Microbiology and Molecular Genetics, College of Medicine, University of California—Irvine, Irvine, California 92697-4025,[1] and Department of Medicine, Lineberger Comprehensive Cancer Center, School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599-7295[2]

Received 8 May 2001/Accepted 25 May 2001

We have developed a model system of human fibrosarcoma cell lines that do or do not possess and express an oncogenic mutant allele of N-*ras*. HT1080 cells contain an endogenous mutant allele of N-*ras*, whereas the derivative MCH603 cell line contains only wild-type N-*ras*. In an earlier study (S. Gupta et al., Mol. Cell. Biol. 20:9294–9306, 2000), we had shown that HT1080 cells produce rapidly growing, aggressive tumors in athymic nude mice, whereas MCH603 cells produced more slowly growing tumors and was termed weakly tumorigenic. An extensive analysis of the Ras signaling pathways (Raf, Rac1, and RhoA) provided evidence for a potential novel pathway that was critical for the aggressive tumorigenic phenotype and could be activated by elevated levels of constitutively active MEK. In this study we examined the role of phosphoinositide 3-kinase (PI 3-kinase) in the regulation of the transformed and aggressive tumorigenic phenotypes expressed in HT1080 cells. Both HT1080 (mutant N-*ras*) and MCH603 (wild-type N-*ras*) have similar levels of constitutively active Akt, a downstream target of activated PI 3-kinase. We find that both cell lines constitutively express platelet-derived growth factor (PDGF) and PDGF receptors. Transfection with tumor suppressor PTEN cDNA into HT1080 and constitutively active PI 3-kinase–CAAX cDNA into MCH603 cells, respectively, resulted in several interesting and novel observations. Activation of the PI 3-kinase/Akt pathway, including NF-κB, is not required for the aggressive tumorigenic phenotype in HT1080 cells. Activation of NF-κB is complex: in MCH603 cells it is mediated by Akt, whereas in HT1080 cells activation also involves other pathway(s) that are activated by mutant Ras. A threshold level of activation of PI 3-kinase is required in MCH603 cells before stimulatory cross talk to the RhoA, Rac1, and Raf pathways occurs, without a corresponding activation of Ras. The increased levels of activation seen were similar to those observed in HT1080 cells, except for Raf and MEK, which were more active than HT1080 levels. This cross talk results in conversion to the aggressive tumorigenic phenotype. This latter observation is consistent with our previous observation that overstimulation of the activity of endogenous members of Ras signaling pathways, activated MEK in particular, is a prerequisite for aggressive tumorigenic growth.

Members of the Ras superfamily are small GTP-binding proteins that function as activating transducers of signaling pathways, whose members are commonly kinases or transcription factors (3, 5). Three members of this family, namely, H-*ras*, K-*ras*, and N-*ras*, have been implicated in human cancers. Mutations in *ras* alleles have been found in more than 30% of human cancers. The mutations invariably result in chronic GTP binding to the Ras molecule and its consequent chronic activation. This state results in constitutive activation of Ras-dependent signaling pathways. Among these are the Raf, Rac1, RhoA, and phosphoinositide (PI) 3-kinase signal transduction cascades (29). These pathways have been shown to regulate mitogenesis signals, motility and invasiveness, actin cytoskeletal architecture, and cell survival, respectively (10, 21, 38, 39). Derangement of the normal regulation of these cellular processes, as occurs when mutant Ras proteins are expressed, is deleterious for the normal behavior of the cells in question and contributes to the progression to a cancerous state.

A variety of experimental procedures, usually utilizing rodent cells, have shown that downstream members of each of the signaling pathways identified above, when mutated, function as transforming oncogenes (23). Among these genes are PI 3-kinase and its downstream target Akt, also known as protein kinase B (2, 41). PI 3-kinase activates Akt, a serine threonine kinase (25), which in turn phosphorylates a number of substrates, including Bad, caspase 9, Forkhead transcription factors, and IKKα (6, 9, 13, 33). Phosphorylation of Bad, procaspase 9, and Forkhead transcription factors inactivates these proapoptotic molecules, whereas phosphorylation of IKKα activates this kinase, leading eventually to activation of the anti-apoptotic NF-κB transcription factor. Each of these substrates is implicated in cell survival. One of the major cell survival factors is NF-κB, whose activation status is dependent upon binding to the IκB protein. The IκB protein complexes with NF-κB and sequesters it in the cytoplasm, thereby preventing it from entering the nucleus. Degradation of IκB, following phosphorylation by IKK, releases NF-κB, which then enters the nucleus and activates its target genes (22, 40, 48). Activation of NF-κB is associated with increased cell survival and cell proliferation (4, 49, 50). One proposed mechanism for the

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, University of California, Irvine, College of Medicine, 240 Med. Sci. Bldg. I, Bldg. B, Irvine, CA 92697-4025. Phone: (949) 824-7042. Fax: (949) 824-8598. E-mail: ejstanbr@uci.edu.

† Present address: Department of Pharmacology and Cancer Biology Duke University Medical Center, Durham, NC 27710.

activation of IKK is phosphorylation mediated by Akt (33, 42). However, other mechanisms also exist that do not involve the degradation of IκB (27, 44).

In addition to being activated by Ras-GTP, PI 3-kinase may also be activated directly by contact with activated growth factor receptors, including platelet-derived growth factor (PDGF) (20, 46). Dysregulated PI 3-kinase activity is likely to play an important role in cancer progression. One indication of this has been the identification of the PTEN tumor suppressor gene (26, 45). PTEN is a common target of inactivating mutations in a variety of sporadic human cancers. In addition, germ line mutations in the PTEN gene are associated with Cowden's disease, an inherited hamartoma syndrome that includes an elevated risk of breast and thyroid cancers (31). The PTEN protein functions as both a protein and a lipid phosphatase. It is the lipid phosphatase activity that is critical for its tumor-suppressing function (30). PTEN lipid phosphatase catalyzes the dephosphorylation of the 3 position of PI 3,4,5-triphosphate (PIP3) and PI 3,4,-biphosphate (PIP2), both of which are the lipid byproducts of the lipid kinase activity of PI 3-kinase. The Akt molecule binds to PIP3 via its pleckstrin homology (PH) domain. In this complex with PIP3, Akt is then phosphorylated and activated by the PI-dependent kinase, PDK-1 (1, 8). Thus, normal cells integrate the activities of PI 3-kinase and PTEN to facilitate homeostasis with respect to PI 3-kinase-mediated signal transduction and cell cycle control. Overactivation of PI 3-kinase or loss of PTEN function is likely to cause dysregulation of this finely balanced control. An illustration of this is that expression of wild-type PTEN transfected into PTEN-null cancer cells results in induction of G1 arrest and/or apoptosis (12, 16). Conversely, this arrest can be overridden by a constitutively active form of Akt (52, 55).

We have developed an experimental model system comprising the human fibrosarcoma cell line HT1080, which possesses one mutant N-*ras* allele, and its derivative, MCH603, which has deleted the mutant allele and possesses only wild-type N-*ras* (35). Examination of these cells has shown that HT1080 has a typical transformed phenotype in culture, including disorganized actin stress fibers and the ability to grow in soft agar, plus an aggressive tumorigenic phenotype in vivo in immunodeficient mice. By contrast, MCH603 cells have "reversed" their transformed phenotype; they have restored a well-organized actin stress fiber distribution in the cytoplasm and are no longer able to grow in soft agar. When implanted into immunodeficient mice they continue to form tumors but with much slower kinetics. We have described these cells as having a weak tumorigenic phenotype (35).

When we examined the activation of a number of Ras signaling pathways, namely, the Raf, Rac1, and RhoA pathways, we found that all members were constitutively active in HT1080 but had basal activity in MCH603 cells (36). However, we noted that Akt was constitutively active in both cell lines. Since this was not due to oncogenic Ras expression in MCH603 cells, we looked for another explanation. In this study we found that both cell lines constitutively synthesize and secrete PDGF and contain cell surface PDGF receptor (PDGFR). Thus, this provides a mechanism for constitutive activation of PI 3-kinase, resulting in the activation of Akt.

Although HT1080 and MCH603 cells have different transformed and tumorigenic phenotypes and yet both have constitutively active Akt, it is formally possible that there may be quantitative and qualitative differences in the activation of PI 3-kinase and/or Akt and their downstream substrates in the two cell lines that play a role in the expression of these phenotypes. In order to determine this, we have modulated the activation of PI 3-kinase and Akt by stable transfection of HT1080 and MCH603 cells with PTEN and an activated mutant of PI 3-kinase (hereafter termed $PI3K^{act}$), respectively. Examination of the biochemical and biological properties of the parental and transfectant cells has revealed several unexpected and novel findings with respect to both signal transduction pathways and biological behavior.

MATERIALS AND METHODS

Molecular constructs. The expression plasmids used in this study were as follows: $PI3K^{act}$-pCMV(hyg)P110CAAX5′myc is derived from pSG5P110 CAAX5′myc (51) and encodes the catalytic domain of PI 3-kinase. The constitutively active protein product, $PI3K^{act}$, is permanently plasma membrane associated. The construct pCDNA3PTEN(wt) (Neo) encodes a full-length wild-type PTEN cDNA (52), whose expression is driven from a heterologous cytomegalovirus promoter.

Cell culture and stable transfection. The HT1080 cell line has one mutant and one wild-type N-*ras* allele (28, 35). MCH603 is a variant of HT1080 and contains only wild-type N-*ras* (35). The cell lines were maintained in Dulbecco minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS; Life Technologies). The HT1080 and MCH603 cell lines were transfected with the PTEN(wt) and $PI3K^{act}$ plasmids, respectively. Clones from each transfection were selected and maintained in medium containing the relevant selective antibiotic (either 800 μg of Geneticin [Gibco-BRL] or 36 U of hygromycin B [Calbiochem] per ml for the HT1080 and MCH603 transfectants, respectively). Subconfluent (70%) 100-mm dishes of MCH603 cells or HT1080 cells were transfected with 5 μg of linearized DNA or vector control DNA, using 30 μl of Lipofectin (Gibco-BRL) in Optimem medium (Gibco-BRL).

Growth in soft agar. Logarithmically growing cells (10^4 or 10^6) were plated in single-cell suspension in a 0.3% top agar overlay in DMEM supplemented with 10% FCS, above a 0.5% bottom agar layer (in DMEM–10% FCS) in 60-mm dishes as previously described (35). Plates were fed periodically with 1 ml of DMEM–10% FCS. Colonies (>0.1 mm) were inspected under the microscope and counted after 3 weeks.

Actin cytoskeleton staining and morphology. Cells grown on glass slides (Nunc) were washed with phosphate-buffered saline (PBS) and fixed with 3.7% paraformaldehyde in PBS for 10 min. After a wash with PBS, cells were permeabilized with PBS containing 0.1% Triton X-100 for 5 min. The slides were then washed, and the actin stress fibers were visualized by staining the cells with fluorescein-conjugated phalloidin (0.005 U/μl; Molecular Probes) for 20 min at room temperature and mounted in ProLong Fade antifade (Molecular Probes).

Immunoblot analyses. Subconfluent cells were serum starved for 18 h, and the cells were then lysed in lysis buffer comprised of 1% sodium dodecyl sulfate (SDS) in 20 mM Tris (pH 7.4), 1 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 0.2 mM sodium orthovanadate. Total cell lysates, each containing 60 μg of protein, were electrophoresed by SDS–7.5% polyacrylamide gel electrophoresis (PAGE) and transferred to Immobilon-P membranes (Millipore). The membranes were then probed with the relevant antibodies. These included PDGFR-α and PDGFR-β (Santa Cruz Biotechnology), Akt/PKB, Phospho-Akt/PKB (Ser473), total Bad, Phospho-Bad, total IκBα, and Phospho-IκBα (New England Biolabs). Following incubation with horseradish peroxidase-conjugated secondary antibody, bound proteins were detected by incubation with a chemiluminescent detection system (Pierce) as previously described (7). In order to test for secreted PDGF in the conditioned medium, subconfluent HT1080 and MCH603 cells were exposed to serum-free medium for 18 h. The conditioned medium was then concentrated in the Centricon (Millipore) apparatus, followed by PAGE under reducing or nonreducing conditions and immunoblotting, using PDGF-A (E-10) and PDGF-B (P-20) antibodies (Santa Cruz Biotechnology).

Activated Ras, Rac1, and RhoA assays. Subconfluent cells were serum starved for 18 h and then lysed with 1 × Mg^{2+} lysis buffer (Ras and Rac Activation Assay Kits; Upstate Biotechnology). Each cell lysate (500 μg) was affinity precipitated with 10 μl of Raf-1 RBD, PAK-1 PBD agarose, or glutathione *S*-transferase (GST)-C21–Sepharose conjugate (43) at 4°C overnight for the Ras, Rac-Cdc42, or RhoA activation assays, respectively. The beads were collected, washed, and resuspended in 6× Laemmli sample buffer. Western blot analysis was performed

as described elsewhere (7), using 1 μg of mouse monoclonal anti-Ras, anti-Rac1 (Upstate Biotechnology), and anti-RhoA (Santa Cruz Biotechnology) antibodies per ml. Horseradish peroxidase-conjugated anti-mouse immunoglobulin G (Santa Cruz Biotechnology) was used as the secondary antibody. A chemiluminescence detection system (Pierce) was used for detection of the relevant proteins. To determine the total Ras, Rac1, or RhoA levels, immunoblots were performed using N-Ras(F155), Rac1(C-14), or RhoA(26C4) antibodies (Santa Cruz Biotechnology) that recognize total protein.

Kinase assays. MEK, ERK, JNK, and Akt kinase assays were performed according to the manufacturer's protocols (New England Biolabs), using subconfluent cultures that had been serum starved (0.25% FCS) for 18 h, and have been described elsewhere (18). Briefly, cells were washed twice with PBS, scraped into 500 μl of lysis buffer, and incubated on ice for 20 min. After centrifugation at 14,000 × *g* for 20 min, the supernatants were incubated with the relevant antibodies. The resulting immunoprecipitates were employed in kinase assays. The activated MEK assay was carried out by incubating immunoprecipitated phospho-MEK with ERK protein and cold ATP (New England Biolabs MEK1/2Kinase Assay Kit). The activated ERK assay was carried out by incubating immunoprecipitated phospho-ERK with Elk-1 fusion protein and cold ATP (New England Biolabs p44/p42 ERK Assay Kit). The JNK assays were carried out by incubating the JNK–c-Jun fusion protein complex with cold ATP (New England Biolabs JNK/SAPK Assay Kit). The Akt-P assay was carried out by incubating the immunoprecipitated total Akt with GSK3 protein and cold ATP (New England Biolabs Akt Assay Kit). For MEK, ERK, and JNK assays, the relevant gel was transferred onto an Immobilon membrane, and Western blot analysis was performed. The blots were performed using phospho-ERK (Thr202/Tyr204) monoclonal antibody for the MEK assay, phospho-Elk-1 (Ser383) polyclonal antibody for the ERK assay, phospho–c-Jun (Ser63) polyclonal antibody for the JNK assay, and phospho-GSK3 α/β (Ser219) rabbit polyclonal antibody for the Akt assay. The Raf-1 assay was performed as described by Graham et al. (17). For the Raf-1 assay, the γ-^{32}P-labeled mitogen-activated protein (MAP) Kinase (ERK) proteins in the gel were visualized by autoradiography. To determine the total Raf, MEK, ERK, JNK, and Akt levels, immunoblots were performed using the respective antibodies that recognize total protein.

Elk-1 and NF-κB luciferase reporter assays. To measure Elk-1 activation, a dual luciferase reporter assay kit (Promega) was used as previously described (18). For NF-κB assays, approximately 2×10^5 parental HT1080 and MCH603 cells and the MCH603/PI3K^{act} or HT1080/PTEN stable transfectant cells were cotransfected in six-well plates with the pUC13-based Δ56FosdE-luc plasmid (measures basal expression but is not Ras responsive) and the NF-κB reporter, $(HIV\text{-}kB)_3$-luc (54). The latter plasmid has three tandem copies of the two adjacent NF-κB sites from the human immunodeficiency virus enhancer (six total tandem NF-κB sites) inserted just upstream of the minimal Fos promoter present in Δ56FosdE-*luc*. The Effectene kit (Qiagen) was used for these transient transfections. Following transfection, the cells were kept in serum-starved medium for 24 h. Tumor necrosis factor alpha (TNF-α; 10 ng/ml) was then added to the culture medium, and both treated and untreated control cultures were incubated for a further 4-h period. The luciferase activity of each sample was measured with the dual luciferase assay kit (Promega) and normalized with an internal control Renilla luciferase.

Tumorigenicity assays. Cells were trypsinized and resuspended in 0.2 ml of DMEM, and then 10^7 cells were injected subcutaneously into the flanks of 4- to 6-week-old nude athymic mice. Tumors were measured in three dimensions with linear calipers at weekly intervals.

RESULTS

We have shown previously that HT1080 (mutant N-*ras*) cells have constitutively active Raf-dependent (Raf/MEK/ERK/Elk-1), Rac1 (Rac1/Cdc42/JNK), and RhoA signaling pathways (18). Conversely, MCH603 (wild-type N-*ras*) cells have basal levels of activity of these signal transduction proteins (18). Interestingly, both HT1080 and MCH603 cells have significant levels of constitutively active Akt. The fact that MCH603 does not possess a mutant *ras* allele and yet has constitutively active levels of Akt, approximating those found in HT1080 cells, infers an alternative mechanism of chronic activation.

HT1080 and MCH603 constitutively secrete PDGF. Concentrated conditioned media and cell lysates from both HT1080 and MCH603 serum-starved cell cultures were electrophoresed and immunoblotted with antibodies to PDGF-A and PDGF-B. Both forms of PDGF were expressed at similar levels by both cell types. However, whereas PDGF-A is secreted into the medium, PDGF-B remains associated with the cells (Fig. 1A). Analyses of PDGF dimers under nonreducing conditions indicated that the predominant secreted form is PDGF-AA (data not shown). Immunoblotting of cell lysates showed that both the α and β forms of the PDGFR are expressed (Fig. 1B). Constitutive secretion of PDGF-A and subsequent binding to and activation of its cognate receptor is, therefore, the probable mechanism for downstream activation of PI 3-kinase and Akt. It is known that the catalytic subunit of PI 3-kinase associates with, and is activated by, the autophosphorylated PDGFR (20, 34, 46).

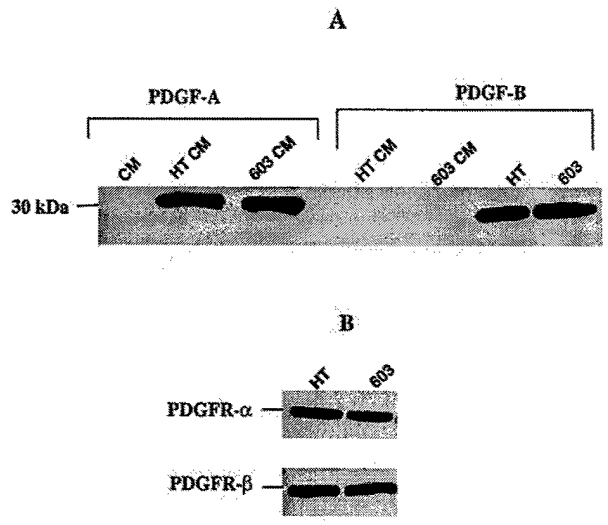


FIG. 1. Western blot analysis performed on HT1080 and MCH603 cell lysates or their respective conditioned media to determine the levels of secreted PDGF-A and PDGF-B (A) or surface membrane-bound PDGFR-α and PDGFR-β (B). HT, HT1080; 603, MCH603; CM, conditioned medium.

Modulation of PI 3-kinase and Akt/PKB activity. (i) HT1080 cells. We wished to downregulate constitutive activity of PI 3-kinase and/or Akt in HT1080 cells. Initially, we attempted downregulation of PI 3-kinase activity via stable transfection with PI 3-kinase dominant-negative cDNAs. Unfortunately, none of the constructs tested (24) had the desired effect (data not shown). Thus, we resorted to expressing the tumor suppressor protein PTEN in these cells. PTEN is a dual-specificity phosphatase that catalyzes the dephosphorylation of PIP3, thereby inhibiting the activation of Akt (30). As shown in Fig. 2A, severalfold-higher levels of expression of PTEN were observed in the HT1080/PTEN stable transfectants, compared to parental HT1080 cells. Correspondingly, there was a decline in the level of expression of activated phospho-Akt. This decline in activity was confirmed in Akt assays (Fig. 3A).

(ii) MCH603 cells. Although these cells already express significant levels of constitutively active Akt, and presumably PI 3-kinase, we wanted to elevate the activity levels even further in order to determine if this may have an effect on in vitro transformed phenotypic traits and in vivo tumorigenicity. To accomplish this, MCH603 cells were stably transfected with a constitutively activated PI 3-kinase–CAAX expression vector (PI3K^{act}) that contains a *myc* epitope tag. As shown in Fig. 2B,

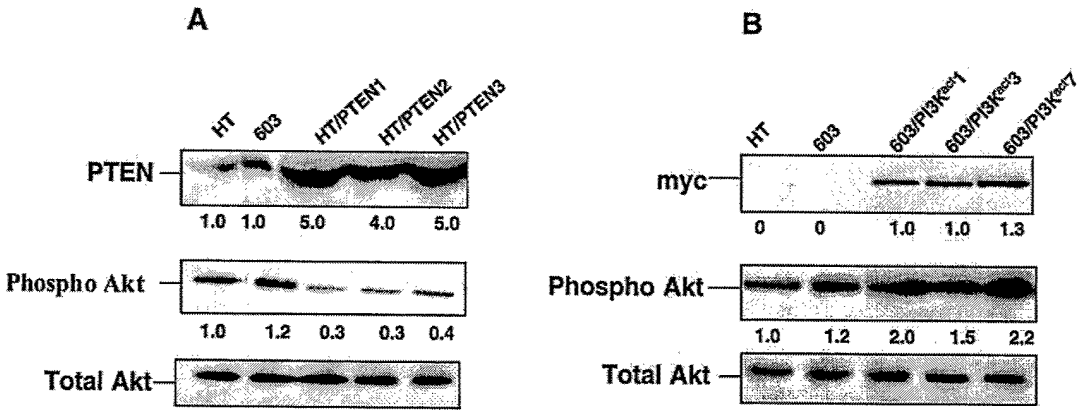


FIG. 2. Western blot analysis of the cell lysates from HT1080/PTEN transfectants (A) and MCH603/PI3K^{act} transfectants (B) to determine the levels of PTEN (A), myc-tagged PI 3-kinase (B), phospho-Akt, and total Akt. Three independent HT1080/PTEN and MCH603/PI3K^{act} clones were analyzed. The fold level of the individual proteins (PTEN and phospho-Akt) is relative to 1.0 for HT1080 control cells. HT, HT1080; 603, MCH603.

the transfectants express high levels of the *myc* epitope tag and, correspondingly, higher levels of activated Akt.

Effects on other Ras-dependent signaling pathways. (i) HT1080/PTEN cells. The lipid phosphatase activity of PTEN dephosphorylates phosphoinositides and would be expected to have inhibitory effects on PI 3-kinase-mediated activation of RhoA-, Rac1-, and Raf-dependent signaling pathways. The protein phosphatase acivity of this dual-specificity phosphatase

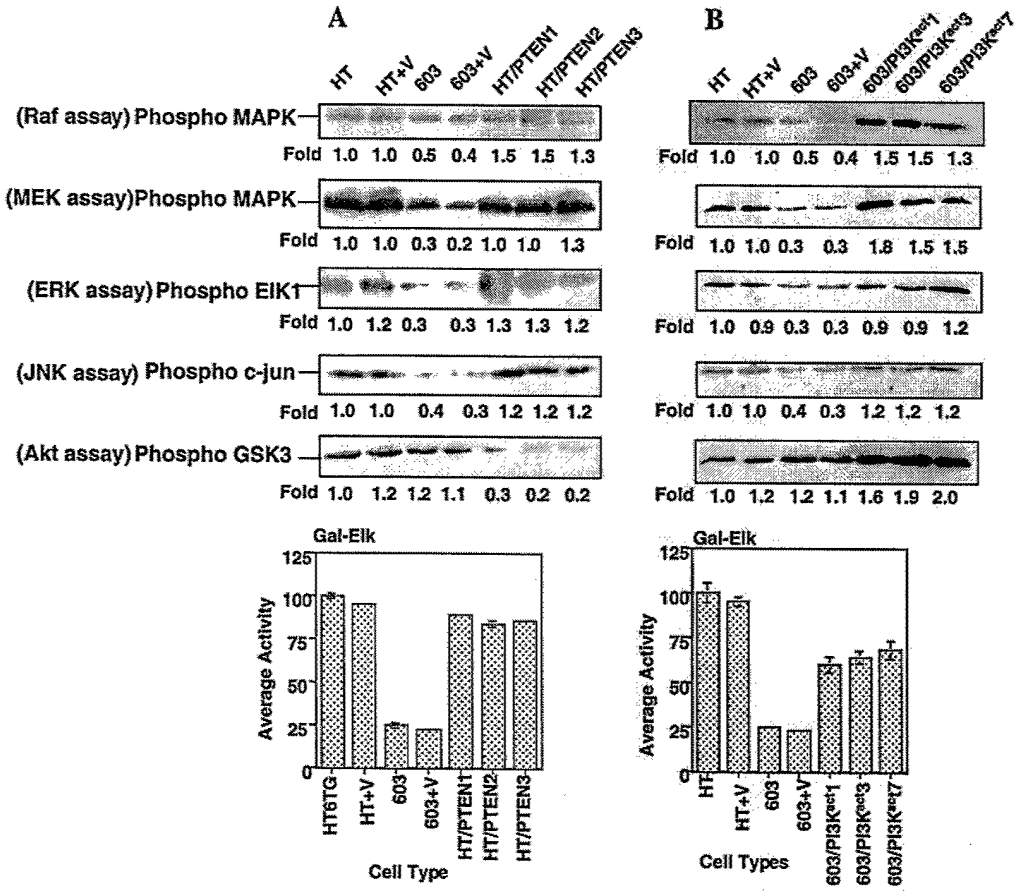


FIG. 3. In vitro Raf, MEK, ERK, and JNK kinase assays and Elk-1 activation assays performed on HT1080/PTEN (A) and MCH603/PI3K^{act} (B) transfectants. For the kinase assays the fold level is relative to 1.0 for HT1080 control cells, and for the Elk-1 luciferase reporter the activities are expressed as the percent relative to 100% for HT1080. Three independent HT1080/PTEN clones and MCH603/PI3K^{act} clones were analyzed. HT, HT1080; 603, MCH603; V, vector only (control). The error bars indicate the standard deviations.

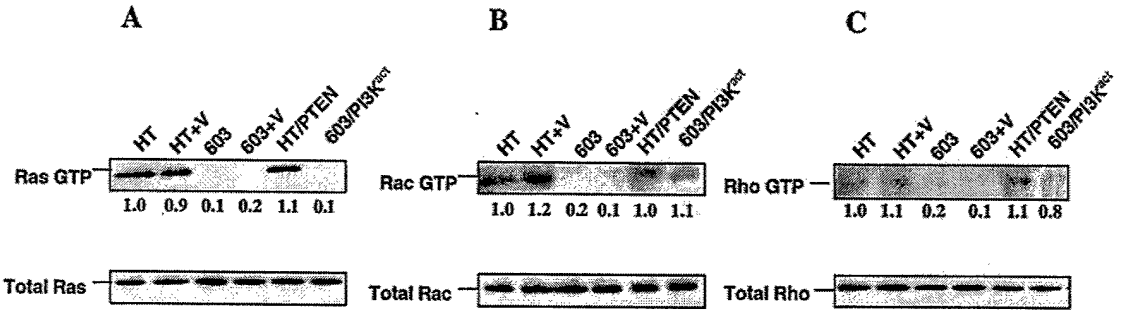


FIG. 4. Pull-down assays of activated Ras, Rac, and Rho. The GTP-bound forms of Ras, Rac, and Rho were pulled down with GST fusion proteins, corresponding to the Ras-binding domain of Raf-1 (Raf-1 RBD), the p21-binding domain (PBD) of human PAK-1, and the C21 binding domain of Rho, respectively, conjugated to agarose beads. The Ras-GTP, Rac-GTP, and Rho-GTP proteins bound to the beads were identified using anti-Ras (A), anti-Rac (B), and anti-Rho (C) antibodies, respectively, in a Western immunoblot. Immunoblot analysis of total cell lysates identified the levels of total protein.

may also have PIP3-independent effects on signal transduction. In the case of the HT1080/PTEN transfectants, however, levels of constitutively active RhoA, Rac1, and JNK and members of the Raf-dependent pathway (Raf/MEK/ERK/Elk-1) remained high, approximating the levels found in parental HT1080 cells (Fig. 3A and 4). These levels of constitutive activity are presumably mediated by the mutant N-Ras protein (see Fig. 3A) in a PI 3-kinase-independent manner.

(ii) MCH603/PI3K^{act} cells. Clear evidence of activation of multiple signaling pathways was found in these cells (Fig. 3B and 4). Persistent activation of RhoA, Rac1, and JNK and members of the Raf-dependent pathway (Raf/MEK/ERK/Elk-1) were observed. However, no activation of Ras was seen (Fig. 4A). Thus, the activation of these pathways was independent of Ras activation and was due either to direct signaling from activated PI 3-kinase or via cross talk between members of the distinct pathways. Quantitation of the levels of activity of the various members of the signaling pathways examined revealed approximately twofold-higher levels of Akt activity in the transfectants, as expected. Levels of activated RhoA and Rac1 approximated that seen in HT1080 cells. A modest but reproducible increase in levels of activated Raf-1 (approximately 1.5-fold) and MEK (1.5- to 1.8-fold) over that seen in HT1080 was observed. The levels of activated ERK and Elk-1 were approximately the same as seen in the HT1080 cells. All levels of constitutive activity were markedly higher than those found in the parental MCH603 cells.

Effects on NF-κB activation. Akt activation, either mediated by PI 3-kinase or other signal transduction pathways, has been shown to be an antiapoptotic survival factor via activation of NF-κB and/or Bad (2, 27, 42). This property may well contribute to the tumor-forming properties of cancer cells. Thus, we wished to determine if activation or downregulation of the activities of these factors affected the tumorigenic phenotypes of HT1080 and MCH603 cells. Akt activation has been reported to activate NF-κB via IκB degradation (33, 42), although other mechanisms of activation have been reported (27, 44). In our studies we examined the status of IκB-α and NF-κB in the parental and transfectant cells.

(i) IκB-α phosphorylation. Degradation of IκB subunits is facilitated by their phosphorylation by IKK (22, 40, 48). Thus, the level of phosphorylated IκB-α, relative to the levels of total IκB-α protein, is indicative of the degradative process. In Fig. 5A we see that HT1080 and MCH603 have comparable levels of phospho-IκB-α relative to the total IκB protein levels. In contrast, the HT1080/PTEN transfectants clones have reduced levels of phospho-IκB-α. Interestingly, the levels of total IκB-α protein increased in these transfectants. Presumably, this is due to the increased stability of the unphosphorylated IκB-α. Thus, lowered Akt activity, mediated by the PTEN lipid phosphatase, results in decreased degradation of IκB-α.

The MCH603/PI3K^{act} transfectants exhibit the opposite characteristics. Increased levels of phospho-IκB-α were seen (Fig. 5B) with correspondingly greatly reduced levels of total

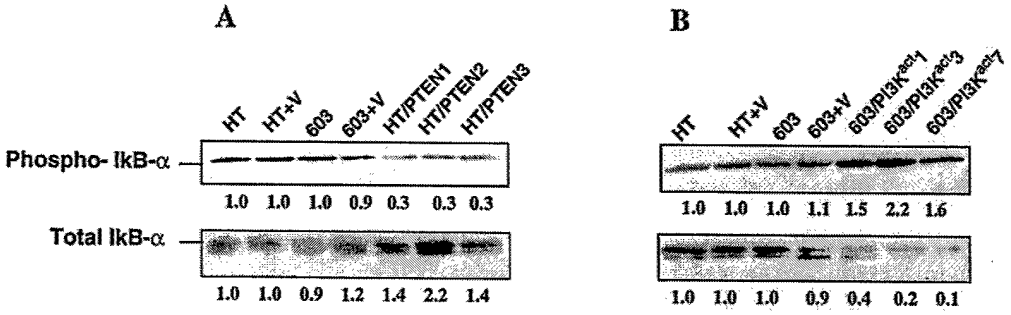


FIG. 5. Western blot analysis performed on HT1080/PTEN transfectants (A) and MCH603/PI3K^{act} (B) transfectants to determine the levels of phospho-IkB and total IkB in these cells.

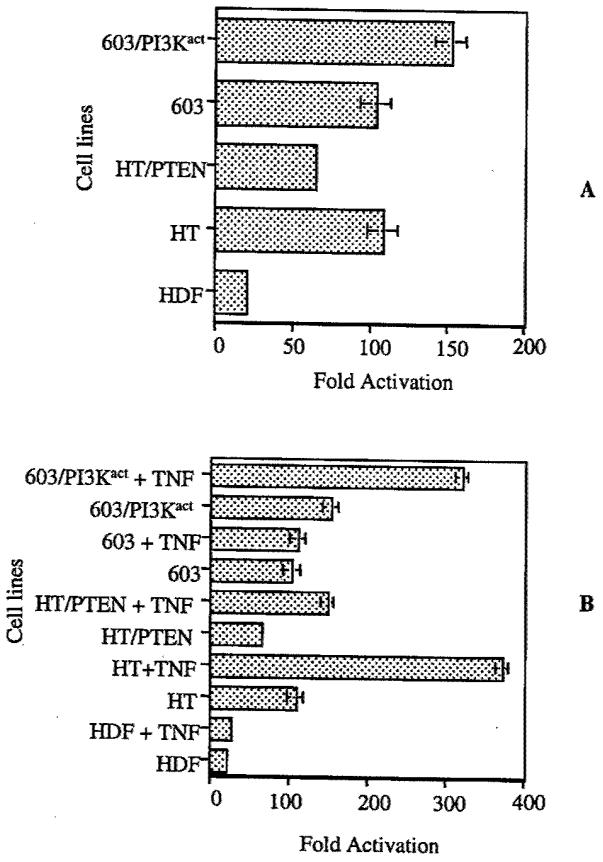


FIG. 6. In vitro luciferase reporter assays were performed to determine NF-κB activities in HT1080, HT1080/PTEN, MCH603, and MCH603/PI3K^{act} cells with (B) or without (A) TNF-α (TNF) treatment. Normal HDFs were used as a control for basal level activity. The NF-κB luciferase reporter activities are presented as the average fold activation. HT, HT1080; 603, MCH603.

IκB-α protein. This is consistent with the increased levels of constitutive Akt activity in these transfectants (Fig. 1B) and is indicative of an increased rate of degradation of IκB-α protein, presumably resulting in a release of IκB-bound NF-κB.

(ii) NF-κB activation. The levels of NF-κB activity in the parental and transfectant cells were assayed, using an NF-κB reporter assay (54). The relative fold activity was determined using an internal control, the Δ56FosdE-luc expression vector.

Consistent with their essentially equal levels of constitutive Akt activity, HT1080 and MCH603 cells had approximately the same fold NF-κB activities. In the HT1080/PTEN transfectants the level of activated NF-κB decreased but did not decline to the level seen in normal human diploid fibroblast (HDF) cells (Fig. 6A). Since the RhoA, Rac1, and Raf signaling pathways remain constitutively active in HT1080 cells, we interpret this to indicate that activation of NF-κB occurs via Akt-dependent and -independent pathways in these cells. In an attempt to clarify this further, we treated the various cell lines with TNF-α, a cytokine that stimulates NF-κB activation via multiple pathways (15, 22). Both HT1080 and HT1080/PTEN NF-κB activity levels were elevated by TNF-α, whereas the level of NF-κB activity in MCH603 cells was unaffected by TNF-α (Fig. 6B). However, the level of NF-κB activity in the MCH603/PI3K^{act} transfectants was substantially increased in the presence of TNF-α (Fig. 6B). It should be noted here that the MCH603/PI3K^{act} cells possess constitutively active RhoA, Rac1, and Raf pathways but not constitutively active Ras (Fig. 3 and 4). Taken together, these data suggest that NF-κB activation in MCH603 cells is Akt dependent, whereas in HT1080 and MCH603/PI3K^{act} cells activation is mediated by both Akt-dependent and independent pathways.

Effects on Bad. Another mechanism whereby activated Akt may function as a survival factor is by phosphorylating the proapoptotic protein Bad, thereby inactivating it and inhibiting the Bad-mediated apoptotic pathway (13). This is, indeed, what was observed: the levels of phosphorylated Bad decreased in the HT1080/PTEN transfectants and increased in the MCH603/PI3K^{act} transfectants, relative to their respective parental cells (Fig. 7).

Biological effects of modulating PI 3-kinase and Akt activity. Activation of PI 3-kinase has been shown to have dramatic effects on the biological behavior of cells, including the transformation of rodent cells (24). We therefore examined a number of phenotypic traits expressed in culture that are associated with neoplastic transformation, plus tumorigenic growth in vivo.

(i) Actin stress fibers. We had earlier shown (18), as is illustrated in Fig. 8A and B, that HT1080 cells have disorganized actin, whereas MCH603 cells have restored an extensive cytoskeleton of actin stress fibers. There was no restoration of actin stress fibers in the HT1080/PTEN transfectants (Fig. 8C). Thus, it appears that phosphoinositide-mediated activation of the Akt pathway is not the determining factor with respect to

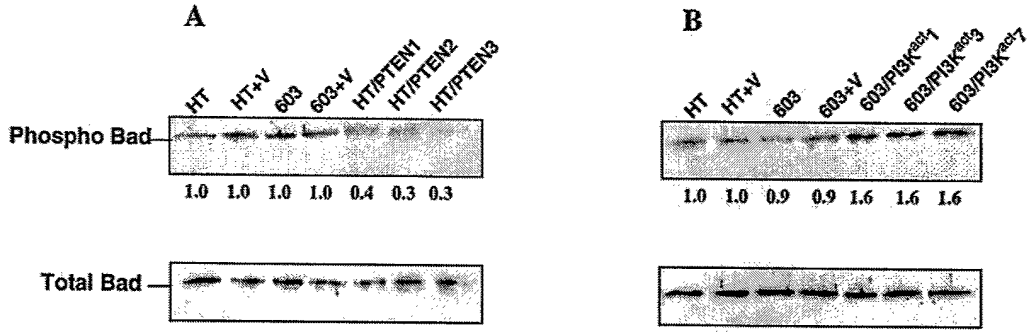


FIG. 7. Western blot analysis performed on HT1080/PTEN transfectants (A) and 603/PI3K^{act} transfectants (B) to determine the level of Phospho-Bad and total Bad in these cells relative to the levels in the parental HT1080 and MCH603 cells.

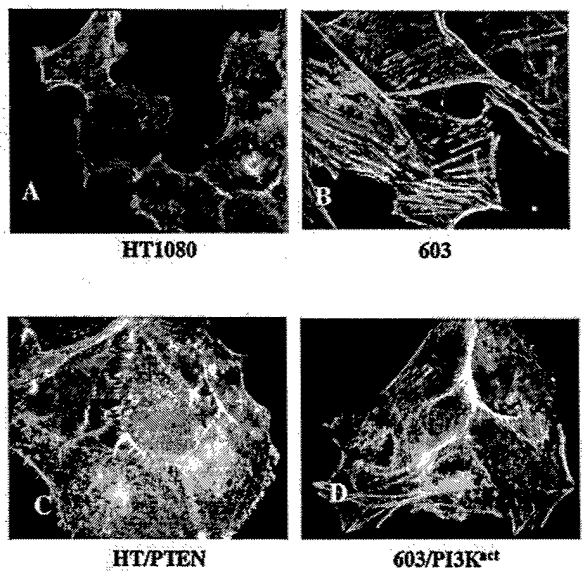


FIG. 8. Actin stress fiber organization in HT1080, MCH603, HT1080/PTEN, and MCH603/PI3K^{act} cells. The cells were stained with fluorescein-conjugated phalloidin. Magnification, ×160.

regulation of actin stress fiber formation. However, as seen in Fig. 8D, increased activation of Akt in the MCH603/PI3K^{act} transfectants is associated with a dramatic loss of actin stress fibers. It should be noted that these cells have also constitutively activated RhoA, Rac1, and Raf/MEK/ERK/Elk-1 signaling pathways (Fig. 3 and 4) and, therefore, more closely resemble HT1080 cells in this regard.

(ii) Anchorage-independent growth. Our earlier studies had shown that HT1080 cells grow well in soft agar, whereas MCH603 cells are incapable of forming colonies in this medium (18). Downregulation of constitutive Akt activity in the HT1080/PTEN transfectants had no effect on this ability to form colonies in soft agar (Fig. 9), whereas MCH603/PI3K^{act} transfectants had a partially restored ability to grow. Colonies were able to form when cells were plated at high density (10^6 cells per dish) but not when plated at low density (10^4 cells per dish). The HT1080 cells form colonies at both plating densities. It should again be noted that the expression of PI3K^{act} in the transfectants activates the RhoA, Rac1, and Raf/MEK/ERK/Elk-1 signaling pathways (Fig. 3 and 4). These same pathways remain constitutively active in the HT1080/PTEN transfectants. Thus, the partial restoration of anchorage-independent growth is not dependent on the constitutive activity of Akt per se but is associated with activation of other Ras-associated signaling pathways.

(iii) Tumor formation. HT1080 and MCH603 cells both form tumors in immune-deficient mice. However, the kinetics of tumor formation differ dramatically. HT1080 cells form aggressively growing tumors that reach a large size within 3 weeks, whereas MCH603 cells form tumors much more slowly. We have termed these phenotypes as aggressive and weak tumorigenic phenotypes, respectively (18, 35). Stable elevated levels of expression of the tumor suppressor protein PTEN in HT1080/PTEN transfectants had no effect on the aggressive tumorigenic phenotype (Fig. 10A). Conversely, elevated levels of activated PI 3-kinase protein in the MCH603/PI3K^{act} transfectants resulted in a conversion from a weak to an aggressive tumorigenic phenotype, albeit not one as aggressive as that seen with HT1080 and HT1080/PTEN cells (Fig. 10B). As with the other biological phenotypes examined, the aggressive and weak tumorigenic phenotypes cannot be a direct consequence of PI 3-kinase or Akt activity. Thus, the antiapoptotic function of NF-κB and inactivation of the proapoptotic factor, Bad, do not seem to influence the aggressive and weak tumorigenic phenotypes of HT1080 and MCH603, respectively. As discussed in more detail below, the activation of MEK in the MCH603/PI3K^{act} transfectants is a likely candidate for orchestrating the conversion from weak to aggressive tumor-forming ability.

DISCUSSION

We have developed an experimental model system that utilizes the HT1080 human fibrosarcoma cell line, possessing a mutant N-*ras* allele, and its derivative, MCH603, in which the mutant N-*ras* allele has been deleted (35). In the HT1080 cells all Ras-dependent pathways examined, namely, the Raf, Rac1, RhoA, and PI 3-kinase/Akt pathways, were constitutively active, presumably as a consequence of the permanent activated

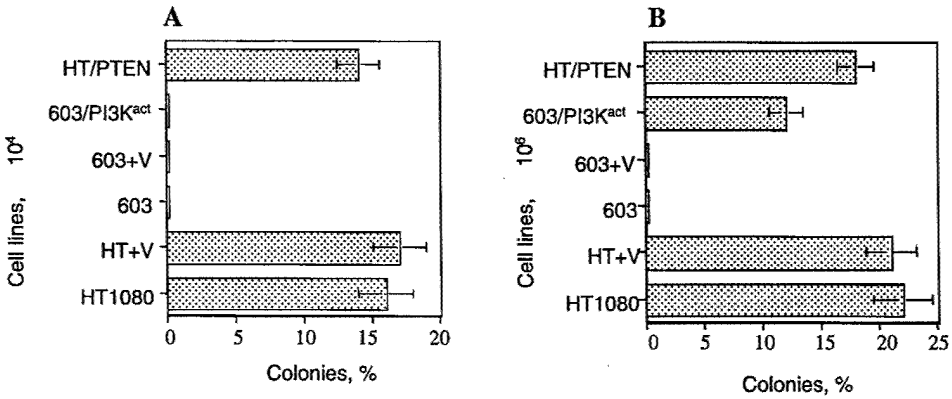


FIG. 9. Anchorage-independent assays. Totals of 10^4 cells (A) or 10^6 cells (B) were plated per 60-mm petri dish in soft agar. Colonies (>0.1 mm) were counted after incubation for 3 weeks at 37°C, with periodic refeeding with fresh growth medium. The error bars indicate standard deviations.

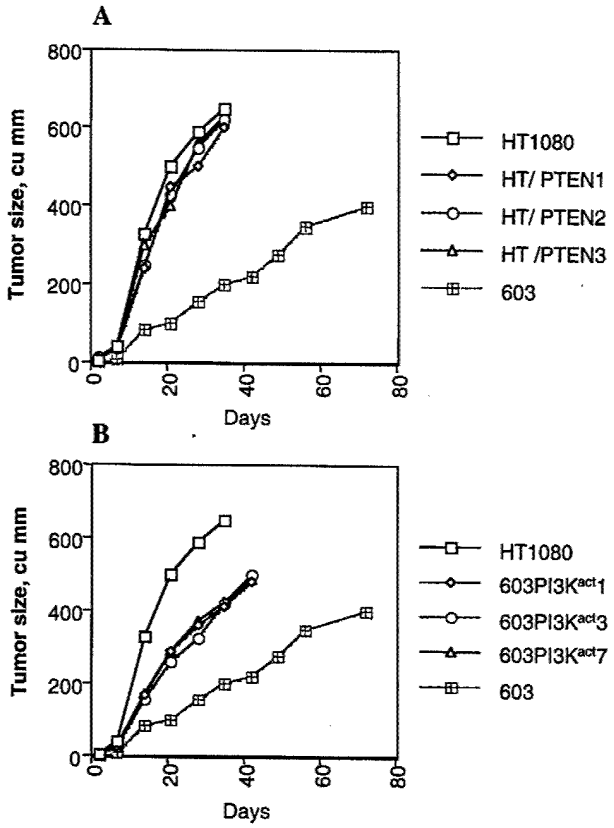


FIG. 10. Tumorigenicity assays of HT1080, MCH603, HT1080/PTEN, and 603/PI3K^{act} cells. Each point is the average size of the tumor sizes of all sites inoculated (total of 6 for the parental cells and 18 for the transfectants, combining three independent clones).

status of the mutant N-Ras protein (18). In contrast, the derivative MCH603 cells exhibit only basal levels of activity of these pathways, with the singular exception of Akt and p38 MAP kinase. We show here that this is probably due to constitutive expression of PDGF and the activation of its cognate PDGFR.

Elevated levels of expression of the lipid phosphatase PTEN protein in HT1080 cells resulted in a significant decrease in activity of Akt. This indicates that the constitutive activation of Akt is mediated via PI 3-kinase generated PIP3, rather than some other pathway, for example Ca^{2+}-calmodulin-dependent protein kinase II (53). Although Akt activity was significantly decreased, the levels of constitutive activity of the RhoA-, Rac1-, and Raf-dependent pathways remained high. Presumably, this is due to the continued stimulation by the endogenous mutant N-Ras protein, whose constitutive activity was unaffected by PTEN.

It is interesting that elevated levels of expression of the PTEN protein did not affect the proliferation of the HT1080/PTEN transfectants, since others have reported that overexpression of PTEN induces G_1 arrest and/or apoptosis (12, 16). However, most of these studies employed transient-transfection methodologies. Also, the cell lines examined were null for PTEN activity (37). Stable transfections of endogenous wild-type PTEN glioma cells with wild-type PTEN cDNA and its subsequent overexpression did not noticeably affect the proliferation of the cells in culture (16). We experienced a similar lack of effect on the growth of HT1080 cells, which are PTEN wild type (data not shown), even though the HT1080/PTEN transfectants express severalfold-higher levels of PTEN protein than the endogenous levels of wild-type PTEN expressed in HT1080. However, the increased levels of PTEN protein did correspond with a decrease in Akt activity. This suggests that the physiological level of endogenous wild-type PTEN in both HT1080 and MCH603 cells did not influence the PIP3-mediated constitutive activation of Akt and further suggests that a threshold level of PTEN protein is required for its inhibitory effect.

Elevating the level of activity of PI 3-kinase in the MCH603/PI3K^{act} transfectants had dramatic effects on the constitutive activities of other putative Ras-dependent pathways examined. The RhoA-, Rac1-, and Raf-dependent pathways were all activated, presumably in an activated PI 3-kinase-dependent fashion involving positive cross talk (47, 51). Interestingly, endogenous Ras was not activated. There has been some debate as to whether low or high levels of activated PI 3-kinase stimulate the activation of Ras (51). In these cells there is clearly no activation of endogenous Ras: thus, PI 3-kinase-mediated activation of these "Ras-dependent" pathways occurs downstream of Ras. It is noteworthy that activation of members of the Raf pathway, in particular MEK, exceeded the levels seen in HT1080 cells even though Ras itself was not activated.

The fact that MCH603 cells have significant levels of Akt activity, which is PI 3-kinase mediated, and yet do not exhibit activation of the RhoA-, Rac1-, and Raf-dependent pathways, suggests that a threshold level of activation is required to initiate the cross talk activation of multiple pathways. Whether this reflects an on/off switch to the activated state, as posited by Ferrell (14), will require further experimentation to determine.

PI 3-kinase-mediated activation of Akt and its subsequent upregulation of the activity of the transcription factor, NF-κB, have been shown to be important modulators of antiapoptotic cell survival (33, 42). Additionally, both Akt and PI 3-kinase, in their activated form, have been shown to have transforming activity in experimental rodent and avian cell systems (2, 11, 24).

Examination of NF-κB activity in the HT1080 and MCH603 parental and HT1080/PTEN and MCH603/PI3K^{act} transfectant cells revealed evidence of complex, multiple pathways of regulation. The complexity of NF-κB activation has been addressed by many investigators. Activation may be effected by oncogenic Ras through Raf-dependent and Raf-independent MAP kinase signaling pathways (15, 19, 32). The Raf-independent pathway appears to signal via Rac and p38 or a closely related kinase. Raf-dependent activation also converges with Raf-independent activation at the level of p38 activation. Furthermore, activation may be effected by PI 3-kinase, either as a consequence of activation of PI 3-kinase by oncogenic Ras or independently of Ras (27, 44).

In the case of the parental HT1080 and MCH603 cells, the basal levels of NF-κB activity were similar and significantly higher than those of normal HDFs. The fact that HT1080 and MCH603 cells have similar levels of constitutive activity of NF-κB under conditions of serum starvation is interesting, given that HT1080 has the capacity to stimulate activity via oncogenic Ras-dependent signaling, as well as PDGF-medi-

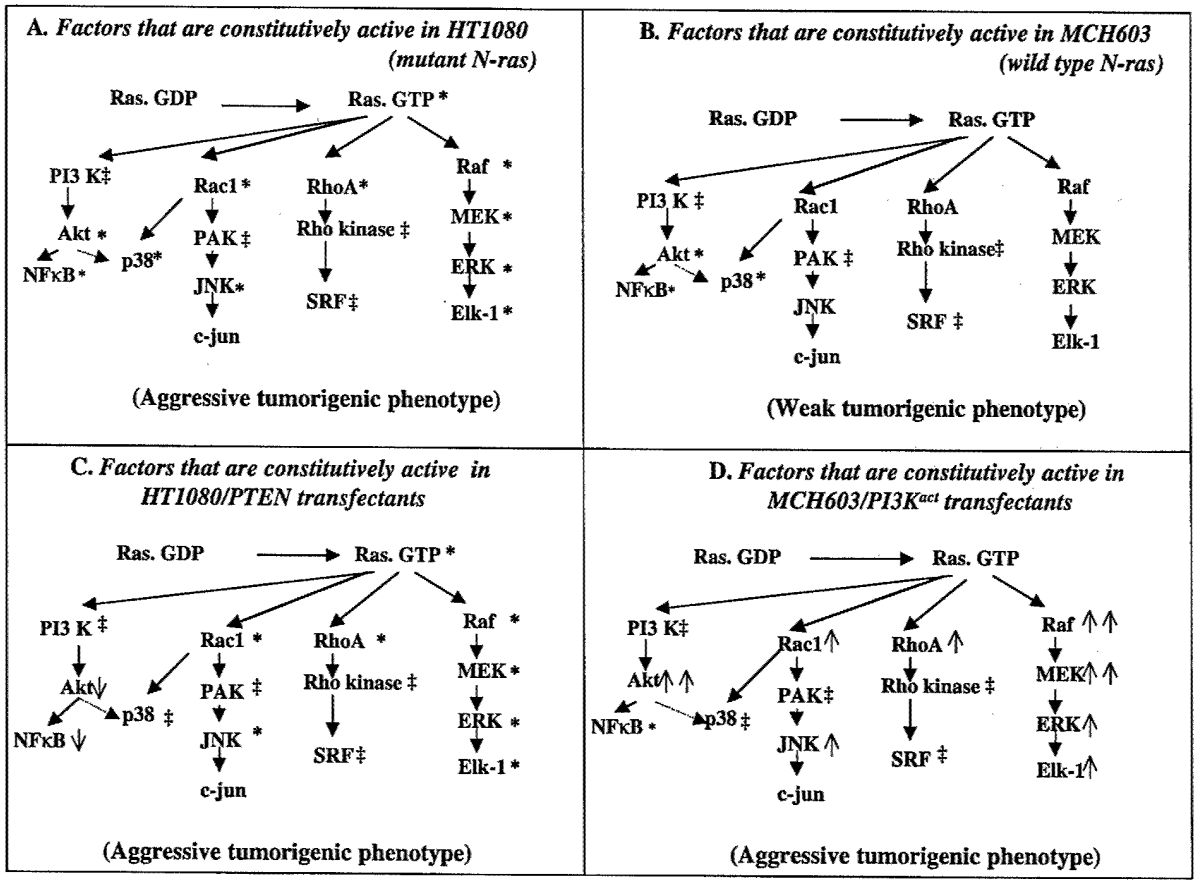


FIG. 11. Summary of levels of constitutive activity of members of the PI 3-kinase, RhoA, Rac1, and Raf signaling pathways and of the tumorigenic phenotypes in parental HT1080 and MCH603 cells and their respective transfectants, HT1080/PTEN and MCH603/PI3K^{act}. *, Proteins constitutively active in HT1080 or MCH603; ↓ or ↑, decrease or increase, respectively, in the constitutive activity of individual factors tested in HT1080/PTEN and MCH603/PI3K^{act} relative to their respective parental cells; ↑ ↑, activity higher than that seen in HT1080 cells; ‡, not tested.

ated PI 3-kinase and Akt signaling, whereas MCH603 cells possess only the latter mechanism. This presumably reflects an upper threshold level of activity under this physiological condition. In both HT1080 and MCH603 cells, activation of NF-κB appears to be associated with, and presumably dependent upon, IκB degradation and the release of NF-κB sequestered in the cytosol. The parental HT1080 and MCH603 cells, however, differ dramatically in their responsiveness to TNF-α stimulation of NF-κB activity. Whereas the activity in HT1080 cells is amplified manyfold, the activity in MCH603 cells is unaltered, as is the case with HDF cells. Thus, it would seem that TNF-α stimulatory effects are mediated only through oncogenic Ras or one or more downstream signaling partners, independently of PI 3-kinase-mediated Akt activation.

Support for this notion is given by the fact that elevated PTEN expression in HT1080/PTEN transfectants reduces the level of constitutive NF-κB activity below that seen in MCH603 cells but not to the level seen in HDFs. This indicates that the constitutive activation of NF-κB in HT1080 is dependent on both PI 3-kinase/Akt and oncogenic Ras signaling. Also, overexpression of activated PI 3-kinase in MCH603/PI3K^{act} transfectants results in levels of constitutive NF-κB activity that are somewhat higher than those seen in MCH603 or HT1080 cells. In these cells exposure to TNF-α does result in an amplification of NF-κB activity to levels approximating those seen in TNF-α-stimulated HT1080 cells. This result is consistent with the observation that the RhoA, Rac1 and Raf signaling pathways all become constitutively activated in these transfectants. It also indicates that Ras-GTP per se is not directly required for TNF-α-mediated stimulation.

A major goal of this study was to determine whether constitutive activation of PI 3-kinase and Akt contributed to the aggressive tumorigenic phenotype of HT1080 fibrosarcoma cells. Our data, which are summarized in Fig. 11, clearly demonstrate that downregulation of this antiapoptotic survival pathway does not demonstrably affect the aggressive tumorigenic phenotype in HT1080/PTEN transfectants. The fact that the HT1080/PTEN transfectants retain the oncogenic Ras-dependent constitutive activation of the RhoA, Rac1, and Raf signaling pathways seems the most likely mechanism for retaining the aggressive tumorigenic phenotype. Consistent with this notion is the observation that overexpression of activated PI 3-kinase in the MCH603/PI3K^{act} transfectants results in constitutive activation of the RhoA-, Rac1-, and Raf-dependent signaling pathways, accompanied by a conversion from the weak to the aggressive tumorigenic phenotype (Fig. 11).

In earlier studies we have shown that, in the absence of mutant N-Ras in the MCH603 cells, overexpression of acti-

vated MEK results in the conversion to an aggressive tumorigenic phenotype (18). This overexpression, coupled with a lack of effect when activated Raf or Rac1 were expressed, led us to speculate that the overexpression of activated MEK in these cells stimulated the activation of a possibly novel pathway that is critical for the conversion to an aggressive tumorigenic phenotype. Consistent with this notion is the observation in this study that the levels of endogenous activated MEK in MCH603/PI3K^{act} transfectants are higher than that seen in HT1080 cells. Thus, the same putative novel pathway may be activated in these cells. Further experimentation is required to test this hypothesis.

The generality of the phenomena described here with respect to other human cancers and cell lines must await further examination. If a novel pathway is confirmed and found to be general for human cancers that express mutant Ras proteins, this may provide an important target for cancer therapy.

ACKNOWLEDGMENTS

We thank Julian Downward and Craig Hauser for the gifts of plasmids and Albert Baldwin, Jr., for critical reading of the manuscript.

These studies were supported by NCI grants CA69515 (E.J.S.) and CA85772 (Y.E.W.) and DOD grant DAMD17-00-1-0037.

REFERENCES

1. **Alessi, D., M. Deak, A. Casamayor, F. Caudwell, N. Morrice, D. Norman, and P. Gaffney.** 1997. 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the *Drosophila* DSTPK61 kinase. Curr. Biol. **7**:776–789.
2. **Aoki, M., B. Osvaldo, A. Bellacosa, P. Tsichlis, and P. K. Vogt.** 1998. The Akt kinase: molecular determinants of oncogenicity. Proc. Natl. Acad. Sci. USA **95**:14950–14955.
3. **Bar-Sagi, D., and A. Hall.** 2000. Ras and Rho GTPases: a family reunion. Cell **103**:227–238.
4. **Beg, A. A,, and D. Baltimore.** 1996. An essential role of NF-kappaB in preventing TNF-alpha induced cell death. Science **274**:782–784.
5. **Boettner, B., and L. Van Aelst.** 1999. Rac and Cdc42 effectors. Prog. Mol. Subcell. Biol. **22**:135–158.
6. **Brunet, A., A. Bonni, M. J. Zigmond, M. Z. Lin, P. Juo, L. S. Hu, M. J. Anderson, K. C. Arden, J. Blenis, and M. E. Greenberg.** 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell **96**:857–868.
7. **Burnett, W. N.** 1981. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
8. **Cantley, L. C., and B. G. Neel.** 1999. New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/Akt pathway. Proc. Natl. Acad. Sci. USA **96**:4240–4245.
9. **Cardone, M. H., N. Roy, H. R. Stennicke, G. S. Salvesen, T. F. Franke, E. J. Stanbridge, S. Frisch, and J. C. Reed.** 1998. Regulation of cell death protease caspase-9 by phosphorylation. Science **282**:1318–1321.
10. **Carpenter, C. L., and L. C. Cantley.** 1996. Phosphoinositide kinases. Curr. Opin. Cell Biol. **8**:153–158.
11. **Chang, H. W., M. Aoki, D. Fruman, K. R. Auger, A. Bellacosa, P. N. Tsichlis, L. C. Cantley, T. M. Roberts, and P. K. Vogt.** 1997. Transformation of chicken cells by the gene encoding the catalytic subunit of PI 3-kinase. Science **276**:1848–1850.
12. **Davies, M. A., D. Koul, H. Dhesi, R. Berman, T. J. McDonnell, D. McConkey, W. K. Yung, and P. A. Steck.** 1999. Regulation of Akt/PKB activity, cellular growth, and apoptosis in prostate carcinoma cells by MMAC/PTEN. Cancer Res. **59**:2551–2556.
13. **del Peso, L., M. Gonzalez-Garcia, C. Page, R. Herrera, and G. Nunez.** 1997. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science **278**:687–689.
14. **Ferrell, J. E., Jr.** 1997. How responses get more switch-like as you move down a protein kinase cascade. Trends Biochem. Sci. **22**:288–289.
15. **Frost, J. A, J. L. Swantek, S. Stippec, M. J. Yin, R. Gaynor, and M. H. Cobb.** 2000. Stimulation of NFkappa B activity by multiple signaling pathways requires PAK1. J. Biol. Chem. **275**:19693–19699.
16. **Furnari, F. B., H. Lin, H. J. S. Huang, and W. K. Cavenee.** 1997. Growth suppression of glioma cells by PTEN requires a functional phosphatase catalytic domain. Proc. Natl. Acad. Sci. USA **94**:12479–12484.
17. **Graham, A., A. McCleese, K. Malarkey, G. W. Gould, and R. Plevin.** 1996. Role of receptor desensitization, phosphatase induction and intracellular cyclic AMP in the termination of mitogen-activated protein kinase activity in UTP-stimulated Eahy 9266 endothelial cells. Biochem. J. **315**:563–569.
18. **Gupta, S., R. Plattner, C. J. Der, and E. J. Stanbridge.** 2000. Dissection of ras-dependent signaling pathways controlling aggressive tumor growth of human fibrosarcoma cells: evidence for a potential novel pathway. Mol. Cell. Biol. **20**:9294–9306.
19. **Jefferies, C. A., and L. A. O'Neill.** 2000. Rac1 regulates interleukin 1-induced nuclear factor kappaB activation in an inhibitory protein kappaBalpha-independent manner by enhancing the ability of the p65 subunit to transactivate gene expression. J. Biol. Chem. **275**:3114–3120.
20. **Jones, S. M., R. Klinghoffer, G. D. Prestwich, A. Toker, and A. Kazlauskas.** 1999. PDGF induces an early and a late wave of PI 3-kinase activity, and only the late wave is required for progression through G1. Curr. Biol. **9**:512–521.
21. **Joneson, T., and D. Bar-Sagi.** 1997. Ras effectors and their role in mitogenesis and oncogenesis. J. Mol. Med. **75**:587–593.
22. **Karin, M., and Y. Ben-Neriah.** 2000. Phosphorylation meets ubiquitination: the control of NF-κB activity. Annu. Rev. Immunol. **18**:621–663.
23. **Khosravi-Far, R., P. A. Solski, G. J. Clark, M. S. Kinch, and C. J. Der.** 1995. Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. Mol. Cell. Biol. **15**:6443–6453.
24. **Klippel, A., M. A. Escobedo, M. S. Wachowicz, G. Apell, T. W. Brown, M. A. Giedlin, W. M. Kavanaugh, and L. T. Williams.** 1998. Activation of phosphatidylinositol 3-kinase is sufficient for cell cycle entry and promotes cellular changes characteristic of oncogenic transformation. Mol. Cell. Biol. **18**:5699–5711.
25. **Klippel, A., W. Kavanaugh, D. Pot, and L. T. Williams.** 1997. A specific product of phosphatidylinositol 3-kinase directly activates the protein kinase Akt through its pleckstrin homology domain. Mol. Cell. Biol. **17**:338–345.
26. **Li, J., C. Yen, D. Liaw, K. Podsypanina, S. Bose, S. I. Wang, J. Puc, C. Miliaresis, L. Rodgers, R. McCombie, S. H. Bigner, B. C. Giovanella, M. Ittmann, B. Tycko, H. Hibshoosh, M. H. Wigler, and R. Parsons.** 1997. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science **275**:1943–1947
27. **Madrid, L. V., C. Y. Wang, D. C. Guttridge, A. J. G. Schottelius, A. S. Baldwin, Jr., and M. W. Mayo.** 2000. Akt suppresses apoptosis by stimulating the transactivation potential of the RelA/p65 subunit of NF-κB. Mol. Cell. Biol. **20**:1626–1638.
28. **Marshall, C. J., A. Hall, and R. A. Weiss.** 1982. A transforming gene present in human sarcoma cell lines. Nature **299**:171–173.
29. **McCormick, F.** 1994. Activators and effectors of *ras* p21 proteins. Curr. Opin. Genet. Dev. **4**:71–76.
30. **Myers, M. P., I. Pass, I. H. Batty, J. Van der Kaay, J. P. Stolarov, B. A. Hemmings, M. H. Wigler, C. P. Downes, and N. K. Tonks.** 1998. The lipid phosphatase activity of PTEN is critical for its tumor suppressor function. Proc. Natl. Acad. Sci. USA **95**:13513–13518.
31. **Nelen, M. R., W. C. van Staveren, E. A. Peeters, M. B. Hassel, R. J. Gorlin, H. Hamm, C. F. Lindboe, J. P. Fryns, R. H. Sijmons, D. G. Woods, E. C. Mariman, G. W. Padberg, and H. Kremer.** 1997. Germline mutations in the PTEN/MMAC1 gene in patients with Cowden disease. Hum. Mol. Genet. **6**:1383–1387.
32. **Norris, J. L., and A. S. Baldwin, Jr.** 1999. Oncogenic Ras enhances NF-κB transcriptional activity through Raf-dependent and Raf-independent mitogen-activated protein kinase signaling pathways. J. Biol. Chem. **274**:13841–13846.
33. **Ozes, O. N., L. D. Mayo, J. A. Gustin, S. R. Pfeffer, L. M. Pfeffer, and D. B. Donner.** 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. Nature **401**:82–85.
34. **Palmer, H. J., V. M. Maher, and J. McCormick.** 1989. Effect of retinoids on growth factor-induced anchorage independent growth of human fibroblasts. J. In Vitro Cell. Dev. Biol. **25**:1009–1015.
35. **Plattner, R., M. J. Anderson, K. Y. Sato, C. L. Fasching, C. J. Der, and E. J. Stanbridge.** 1996. Loss of oncogenic *ras* expression does not correlate with loss of tumorigenicity in human cells. Proc. Natl. Acad. Sci. USA **93**:6665–6670.
36. **Plattner, R., S. Gupta, R. Khosravi-Far, K. Y. Sato, M. Perucho, C. J. Der, and E. J. Stanbridge.** 1999. Contribution of the ERK and JNK mitogen-activated protein kinase cascades to Ras transformation of HT1080 fibrosarcoma and DLD-1 colon carcinoma cells. Oncogene **18**:1807–1817.
37. **Ramaswamy, S., N. Nakamura, F. Vazquez, D. B. Batt, S. Perera, T. M. Roberts, and W. R. Sellers.** 1999. Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. Proc. Natl. Acad. Sci. USA **96**:2110–2115.
38. **Ridley, A. J., and A. Hall.** 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell **70**:389–99.
39. **Ridley, A. J., H. F. Paterson, C. L. Johnston, D. Diekmann, and A. Hall.** 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell **70**:401–410.
40. **Rodriguez, M. S., J. Wright, J. Thompson, D. Thomas, F. Baleux, J. L. Virelizier, R. T. Hay, and F. Arenzana-Seisdedos.** 1996. Identification of

lysine residues required for signal-induced ubiquitination and degradation of I kappa B-alpha in vivo. Oncogene **12:**2425–2435.
41. **Rodriguez-Viciana, P., P. H. Warne, A. Khwaja, B. M. Marte, D. Pappin, P. Das, M. D. Waterfield, A. Ridley, and J. Downward.** 1997. Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. Cell **89:**457–467.
42. **Romashkova, J. A., and S. S. Makarov.** 1999. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. Nature **401:**86–90.
43. **Sander, E. E., S. Van Delft, J. P. Ten Klooster, T. Reid, R. A. Van der Kammen, F. Michiels, and J. G. Collard.** 1998. Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. J. Cell Biol. **143:**1385–1398.
44. **Sizemore, N., S. Leung, and G. R. Stark.** 2000. Activationof phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-κB p65/RelA subunit. Mol. Cell. Biol. **19:**4798–4805.
45. **Steck, P. A., M. A. Pershouse, S. A. Jasser, W. K. Yung, H. Lin, A. H. Ligon, L. A. Langford, M. L. Baumgard, T. Hattier, T. Davis, C. Frye, R. Hu, B. Swedlund, D. H. Teng, and S. V. Tavtigian.** 1997. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. Nat. Genet. **15:**356–362.
46. **Susa, M., M. Keeler, and L. Varticovski.** 1992. Platelet-derived growth factor activates membrane-associated phosphatidylinositol 3-kinase and mediates its translocation from the cytosol. Detection of enzyme activity in detergent-solubilized cell extracts. J. Biol. Chem. **267:**22951–22956.
47. **Tang, Y., J. Yu, and J. Field.** 1999. Signals from the Ras, Rac, and Rho GTPases converge on the Pak protein kinase in Rat-1 fibroblasts. Mol. Cell. Biol. **19:**1881–1891.
48. **Traenckner, E. B., H. L. Pahl, T. Henkel, K. N. Schmidt, S. Wilk, and P. A. Baeuerle.** 1995. Phosphorylation of human I kappa B-alpha on serines 32 and 36 controls I kappa B-alpha proteolysis and NF-kappa B activation in response to diverse stimuli. EMBO. J. **14:**2876–2883.
49. **Van Antwerp, D. J., S. J. Martin, T. Kafri, D. R. Green, and I. M. Verma.** 1996. Suppression of TNF-alpha-induced apoptosis by NF-kappa-B. Science **274:**787–789.
50. **Wang, C. Y., M. W. Mayo, and A. S. Baldwin, Jr.** 1996. TNF-alpha and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. Science **274:**784–787.
51. **Wennstrom, S., and J. Downward.** 1999. Role of phosphoinositide 3-kinase in activation of ras and mitogen-activated protein kinase by epidermal growth factor. Mol. Cell. Biol. **6:**4279–4288.
52. **Wu, X., K. Senechal, M. S. Neshat, Y. E. Whang, and C. L. Sawyers.** 1998. The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. Proc. Natl. Acad. Sci. USA **95:**15587–15591.
53. **Yano, S., H. Tokumitsu, and T. R. Soderling.** 1998. Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. Nature **39:**584–587.
54. **Ye, X., P. Mehlen, S. Rabizadeh, T. VanArsdale, H. Zhang, H. Shin, J. J. Wang, E. Leo, J. Zapata, C. A. Hauser, J. C. Reed, and D. E. Bredesen.** 1999. TRAF family proteins interact with the common neurotrophin receptor and modulate apoptosis induction. J. Biol. Chem. **274:**30202–30208.
55. **Zhang, P., and B. M. Steinberg.** 2000. Overexpression of PTEN/MMAC1 and decreased activation of Akt in human papillomavirus-infected laryngeal papillomas. Cancer Res. **60:**1457–1462.

Oncogene (2002) 21, 319–327

www.nature.com/onc

PTEN sensitizes prostate cancer cells to death receptor-mediated and drug-induced apoptosis through a FADD-dependent pathway

Xiu-Juan Yuan[1] and Young E Whang*[,1,2]

[1]*Lineberger Comprehensive Cancer Center, University of North Carolina School of Medicine, Chapel Hill, North Carolina, NC 27599-7295, USA;* [2]*Department of Medicine, Division of Hematology-Oncology, University of North Carolina School of Medicine, Chapel Hill, North Carolina, NC 27599-7295, USA*

The PTEN tumor suppressor is frequently mutated in human tumors. Loss of PTEN function is associated with constitutive survival signaling through the phosphatidylinositol-3 kinase/Akt pathway. Therefore, we asked if reconstitution of PTEN function would lead to the reversal of resistance to apoptosis in prostate cancer cells. Adenovirus-mediated expression of PTEN completely suppressed constitutive Akt activation in LNCaP prostate cancer cells and enhanced apoptosis induced by a broad range of apoptotic stimuli. PTEN expression sensitized cells to death receptor-mediated apoptosis induced by tumor necrosis factor, anti-Fas antibody, and TRAIL. PTEN also sensitized cells to non-receptor mediated apoptosis induced by a kinase inhibitor staurosporine and chemotherapeutic agents mitoxantrone and etoposide. PTEN-mediated apoptosis was accompanied by caspase-3 and caspase-8 activation and was inhibited by a broad specificity caspase inhibitor Z-VAD-fmk. Bcl-2 overexpression also blocked PTEN-mediated apoptosis. Lipid phosphatase activity of PTEN is required for apoptosis as the PTEN G129E mutant selectively deficient in lipid phosphatase activity was unable to sensitize cells to apoptosis. PTEN-mediated apoptosis involves a FADD-dependent pathway for both death receptor-mediated and drug-induced apoptosis as coexpression of a dominant negative FADD mutant blocked PTEN-mediated apoptosis. Since in death receptor signaling, FADD mediates activation of caspase-8, which in turn cleaves BID, and since caspase-8 is activated in PTEN-mediated apoptosis, we examined BID cleavage in PTEN-mediated apoptosis. PTEN facilitated BID cleavage after treatment with low doses of staurosporine and mitoxantrone. BID cleavage was inhibited by dominant negative FADD. Taken together, these data are consistent with the hypothesis that PTEN promotes drug-induced apoptosis by facilitating caspase-8 activation and BID cleavage through a FADD-dependent pathway.
Oncogene (2002) **21**, 319–327. DOI: 10.1038/sj/onc/1205054

Keywords: PTEN; apoptosis; FADD; death receptors

*Correspondence: YE Whang; E-mail: ywhang@med.unc.edu
Received 29 May 2001; revised 25 September 2001; accepted 9 October 2001

Introduction

The *PTEN* tumor suppressor (also known as *MMAC1*) is one of the most frequently mutated genes in human malignancies and is inactivated in a wide range of tumors, including melanoma and cancers of the brain, endometrium, and prostate (Li *et al.*, 1997a; Steck *et al.*, 1997). Mice with heterozygous disruption of *PTEN* are predisposed to develop multiple types of tumors (Di Cristofano *et al.*, 1998; Podsypanina *et al.*, 1999; Suzuki *et al.*, 1998a). The PTEN protein, through its ability to dephosphorylate the lipid second messenger phosphatidylinositol (PI) 3,4,5-phosphate, negatively regulates survival signaling mediated by the PI 3-kinase/Akt pathway (Maehama and Dixon, 1998). Loss of PTEN in tumor cells leads to persistent activation of the serine/threonine kinase Akt (Myers *et al.*, 1998; Stambolic *et al.*, 1998; Wu *et al.*, 1998).

Two major pathways leading to apoptosis have been elucidated (Budihardjo *et al.*, 1999). In the mitochondrial dependent pathway, stimuli such as stress, withdrawal of survival factors, DNA damage, or chemotherapeutic agents cause release of cytochrome *c* from mitochondria leading to the formation of 'apoptosomes' consisting of cytochrome *c*, Apaf-1, and procaspase-9 (Li *et al.*, 1997b). This results in autoactivation of procaspase-9 and subsequently activation of effector caspases and a distinct apoptotic cell death program. Another pathway involves the death signal generated at the cell membrane by receptors such as tumor necrosis factor (TNF)-α receptor and Fas (Ashkenazi and Dixit, 1999). Binding of ligands to death receptors initiates recruitment and assembly of the death-inducing signaling complex, consisting of receptor cytoplasmic domains, adaptor proteins such as TRADD (TNF receptor associated death domain) and FADD (Fas associated death domain), and procaspase-8. Activated caspase-8 may directly activate downstream effector caspases in some cells or alternately in other cell types the death signal may be amplified through mitochondria by cleavage of the proapoptotic Bcl-2 family member BID, which translocates to mitochondria and induces cytochrome *c* release from mitochondria and thereby leads to activation of downstream caspases (Li *et al.*, 1998; Luo *et al.*, 1998). Signaling networks initiated by growth factor and cytokine receptors regulate apopto-

sis at multiple points and more refined understanding of how tumor suppressors such as PTEN regulate sensitivity to apoptosis may lead to more specific targeting of tumor cells by cancer therapeutic agents.

Prostate cancer is the second leading cause of cancer-related mortality in American men. Molecular mechanisms underlying development and progression of prostate cancer remain incompletely understood. PTEN function is lost in a high percentage of both localized and advanced prostate cancer tumors and this is associated with constitutive Akt activation (McMenamin *et al.*, 1999; Suzuki *et al.*, 1998b; Wu *et al.*, 1998). Therefore, we asked if reconstitution of PTEN function would lead to the reversal of resistance to apoptosis in prostate cancer cells. We show that PTEN expression sensitize prostate cancer cells to multiple apoptotic stimuli in a caspase-dependent manner and that lipid phosphatase activity of PTEN is required for this function and that FADD-dependent signaling is involved in this process, both in death receptor-mediated and drug-induced apoptosis.

Results

Adenovirus-mediated PTEN expression suppresses constitutive Akt activation

Loss of PTEN function in cancer cells leads to constitutive survival signaling through the PI 3-kinase/Akt pathway (Myers *et al.*, 1998; Stambolic *et al.*, 1998; Wu *et al.*, 1998). In order to test the hypothesis that reconstitution of PTEN expression in prostate cancer cells will restore the sensitivity to apoptotic stimuli, we used an adenovirus vector to transduce PTEN expression efficiently into PTEN-null LNCaP prostate cancer cells. As previously reported, LNCaP cells expressed phosphorylated, constitutively activated Akt, but no endogenous PTEN protein (Wu *et al.*, 1998). Reconstitution of PTEN expression by adenoviral transduction completely suppressed phospho-Akt without affecting the total level of Akt (Figure 1a). At 24 h after infection, PTEN was expressed at a level comparable to endogenous levels and at 48 h after infection, PTEN was modestly overexpressed (Figure 1b).

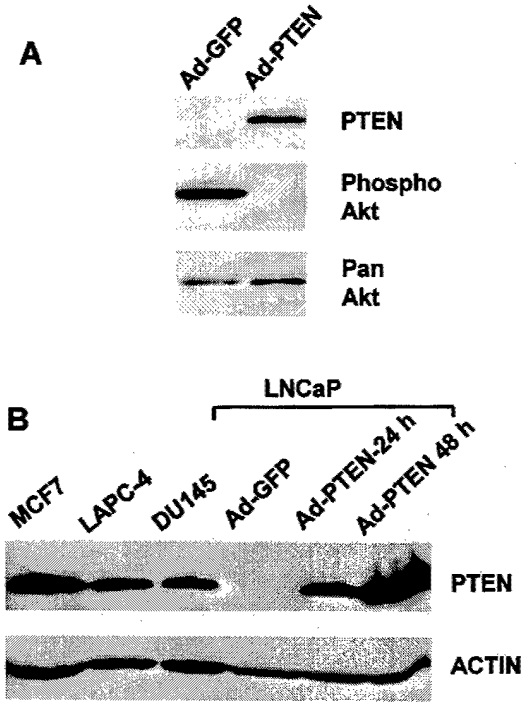


Figure 1 Adenovirus-mediated PTEN expression suppresses constitutive Akt activation. (a) LNCaP cells were infected with Ad-GFP or Ad-PTEN virus at multiplicity of infection (m.o.i.) of 10. PTEN, phosphorylated Akt, and total Akt levels were determined by immunoblotting 48 h after infection. (b) Expression levels of PTEN in MCF7 breast cancer cells, LAPC-4 prostate cancer cells (Klein *et al.*, 1997), DU145 prostate cancer cells, LNCaP cells infected with Ad-GFP and Ad-PTEN, harvested at 24 or 48 h after infection, were determined by immunoblotting. Equivalent loading was confirmed by immunoblotting with anti-actin antibody

PTEN sensitizes cells to both death receptor-mediated and drug-induced apoptosis

Adenoviral PTEN expression by itself induced apoptosis and inhibited growth of LNCaP cells when assayed 4 days after infection (data not shown), in agreement with a previous report (Davies *et al.*, 1999). However, Ad-PTEN did not induce apoptosis in DU145 prostate cancer cells, which express functional PTEN (data not shown). We examined the effect of PTEN expression on the sensitivity to apoptotic stimuli at an early time point. At this time point, PTEN expression by itself had a minimal effect on apoptosis (Figure 2). Ad-GFP infected LNCaP cells were insensitive to treatment with TNF. LNCaP cells require treatment with cycloheximide for induction of apoptosis by TNF (Kulik *et al.*, 2001) (also data not shown). However, Ad-PTEN markedly enhanced induction of apoptosis by TNF. Cells infected with Ad-PTEN and treated with TNF became detached and appeared nonviable (Figure 2). Apoptotic cell death was confirmed in these cells by the appearance of the cell population with the hypodiploid sub-G1 DNA content and also by a DNA fragmentation ELISA assay measuring the amount of DNA-histone complexes released into the cytoplasm. A dose of TNF as low as 1 ng/ml efficiently induced apoptosis in Ad-PTEN infected cells whereas TNF at a dose of up to 100 ng/ml did not induce apoptosis in control adenovirus infected cells (Figure 3a and data not shown). In addition to TNF, PTEN expression sensitized LNCaP cells to apoptosis induced by activation of other death receptors such as Fas and TRAIL (TNF-related apoptosis inducing ligand) receptor. Agonistic anti-Fas antibody induced apoptosis more potently in Ad-PTEN infected LNCaP cells compared to control cells (Figure 3b). Similar sensitization to apoptosis induced by TRAIL could be demonstrated in Ad-PTEN

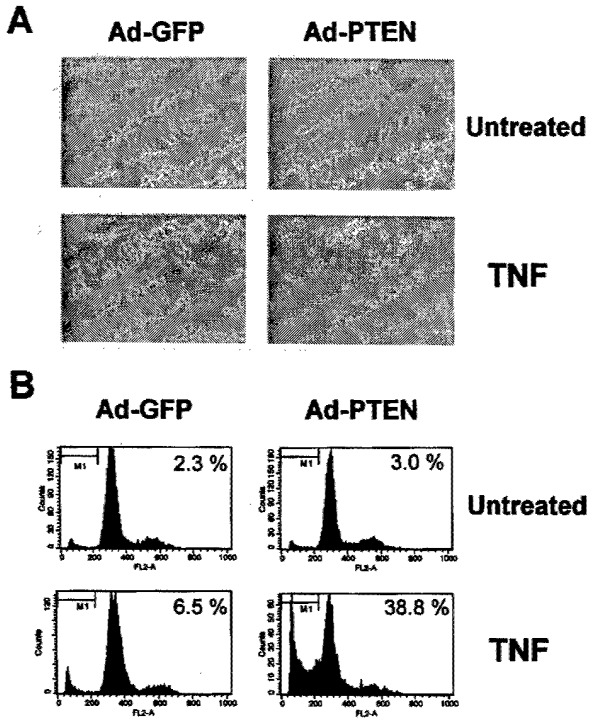


Figure 2 PTEN sensitizes cells to TNF-induced apoptosis. (**a**) Cell morphology by phase-contrast microscopy after adenovirus infection and treatment with TNF. LNCaP cells were infected with Ad-GFP or Ad-PTEN and the next day TNF (40 ng/ml) was added. The morphology of cells is shown after 48 h of treatment. (**b**) Appearance of the apoptotic hypodiploid population after PTEN expression and TNF treatment. LNCaP cells were infected with Ad-GFP or Ad-PTEN and the next day TNF (40 ng/ml) was added. After 24 h of TNF treatment, DNA content was analysed by flow cytometry. The percentage of cells with the hypodiploid DNA content, indicated by the 'M1' marker, is shown

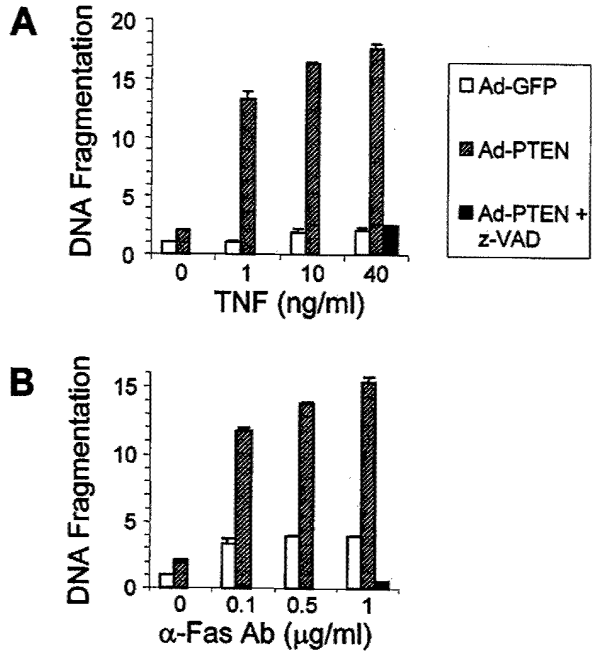


Figure 3 PTEN sensitizes cells to death receptor-mediated apoptosis in a caspase dependent manner. LNCaP cells were infected with Ad-GFP or Ad-PTEN on day 0. On day 1, cells were treated with following apoptotic stimuli. (**a**) TNF for 24 h. The caspase inhibitor, z-VAD-fmk (50 μM), was added at the same time, as indicated. (**b**) Agonistic anti-Fas antibody IPO-4 for 24 h. z-VAD-fmk (25 μM) was added at the same time, as indicated. The extent of apoptosis occurring with each treatment was determined by quantitation of DNA fragmentation with an ELISA assay. Results are shown relative to the background level of apoptosis occurring in untreated Ad-GFP infected cells

infected LNCaP cells (data not shown), in agreement with a recent report (Thakkar *et al.*, 2001). PTEN also sensitized cells to apoptosis not initiated by death ligands (Figure 4). Ad-PTEN infected cells became sensitized to apoptosis induced by staurosporine, a broad spectrum protein kinase inhibitor widely used to induce apoptosis. Mitoxantrone is a chemotherapeutic agent structurally related to adriamycin and is commonly used for treatment of metastatic prostate cancer. Ad-PTEN infected cells were more sensitive to mitoxantrone-induced apoptosis. Similar results were obtained with another chemotherapeutic agent etoposide (data not shown). These data show that PTEN expression lowers the threshold for apoptosis induced by both death ligands and drugs.

PTEN-mediated apoptosis involves caspases-3 and -8 activation and is inhibited by caspase inhibitor

Apoptosis is generally accompanied by activation of caspases. PTEN expression and a second apoptotic stimulus such as TNF, anti-Fas antibody or staurosporine led to marked activation of the DEVD-peptide specific caspase 3-like activity whereas PTEN expression or the apoptotic stimulus by itself induced caspase-3 activity only minimally (Figure 5a). Caspase-3 activity was inhibited by treatment with a broad specificity caspase inhibitor z-VAD-fmk. In addition, z-VAD-fmk efficiently blocked apoptosis induced by PTEN and a second apoptotic stimulus (Figures 3 and 4). Since death receptor signaling involves caspase-8, activation of caspase-8 was examined (Figure 5b). PTEN expression in combination with a second apoptotic stimulus such as TNF, anti-Fas antibody or staurosporine led to activation of procaspase-8 as evidenced by appearance of cleaved caspase-8 isoforms, p43 and p41, on immunoblotting. TNF and anti-Fas antibody by itself (but not staurosporine) stimulated caspase-8 activation to a minimal extent. These data show that PTEN-mediated apoptosis involves caspase activation.

Lipid phosphatase activity of PTEN is required for apoptosis

PTEN can dephosphorylate both protein and lipid substrates (Maehama and Dixon, 1998; Myers *et al.*, 1997). Although much evidence links its tumor suppressive properties to its lipid phosphatase activity, some studies implicate lipid phosphatase-independent

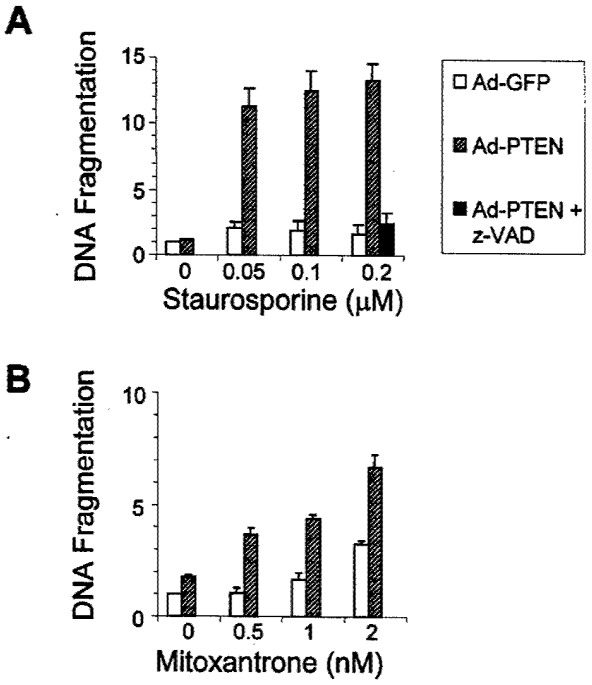


Figure 4 PTEN sensitizes cells to drug-induced apoptosis. LNCaP cells were infected with adenovirus and then treated with the following. (**a**) Staurosporine for 24 h. z-VAD-fmk (25 μM) was added at the same time, as indicated. (**b**) Mitoxantrone for 24 h. The extent of apoptosis was quantified by the DNA fragmentation ELISA assay

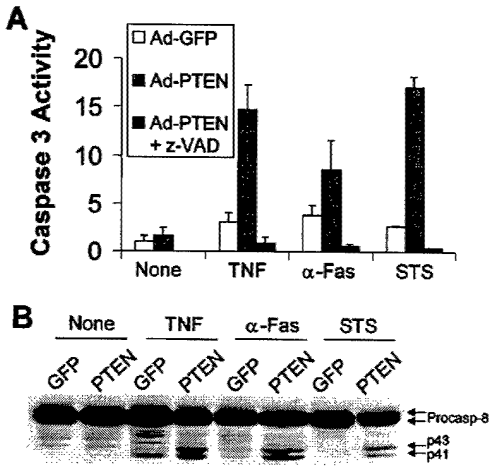


Figure 5 PTEN-mediated apoptosis is accompanied by caspase-3 and caspase-8 activation. LNCaP cells were infected with Ad-GFP or Ad-PTEN and then treated with TNF (40 ng/ml), agonistic anti-Fas antibody IPO-4 (1 μg/ml) or staurosporine (0.1 μM). Cells were treated with the caspase inhibitor z-VAD-fmk (25 μM), as indicated. (**a**) After 24 h of treatment, cell extracts were assayed for caspase 3-like activity by their ability to cleave the DEVD-pNA colorimetric substrate. The specific activity was normalized to untreated Ad-GFP-infected LNCaP cells. (**b**) Lysates from cells treated as above were immunoblotted with anti-caspase 8 specific monoclonal antibody. Unprocessed procaspase-8a/b as well as processed p43 and p41 forms of caspase-8 are indicated by arrows

pathways in PTEN function (Hlobilkova *et al.*, 2000; Maier *et al.*, 1999; Weng *et al.*, 2001). To assess the requirement for lipid phosphatase activity in sensitizing cells to apoptosis, we constructed an adenovirus vector expressing the PTEN G129E mutant and tested its ability to sensitize cells to apoptosis. The PTEN G129E mutant is selectively defective in lipid phosphatase activity but retains tyrosine phosphatase activity (Myers *et al.*, 1998). PTEN G129E, although expressed at a comparable level to wild type PTEN, was unable to suppress Akt activation (Figure 6a). Furthermore, it was completely inactive in sensitizing LNCaP cells to apoptosis induced by TNF (Figure 6b) as well as agonistic anti-Fas antibody and staurosporine (data not shown). These data strongly suggest that PTEN-mediated apoptosis is completely dependent on the lipid phosphatase activity of PTEN.

Bcl-2 blocks PTEN-mediated apoptosis

In some cell types, death receptor signaling proceeds through caspase-8 mediated cleavage of BID, which then translocates to mitochondria and induces the release of cytochrome *c* from mitochondria and subsequent activation of caspases (Li *et al.*, 1998; Luo *et al.*, 1998). In these so-called type II cells, Bcl-2 overexpression blocks the release of cytochrome *c* and

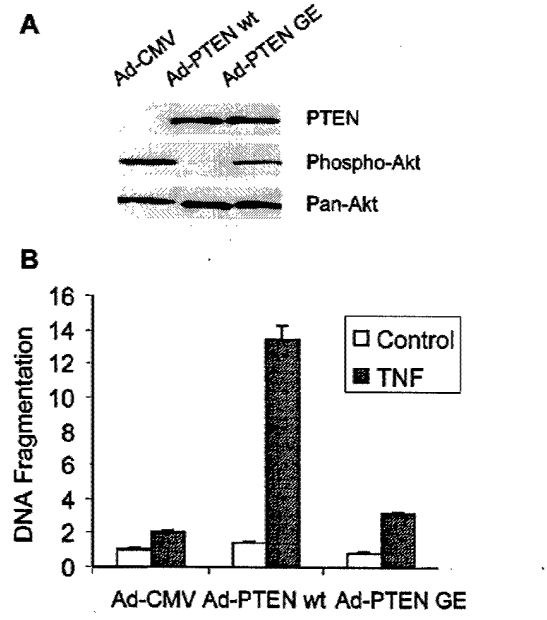


Figure 6 PTEN-mediated apoptosis is dependent on lipid phosphatase activity of PTEN. LNCaP cells were infected with one of following recombinant adenoviruses: Ad-CMV containing the CMV promoter but no transgene, Ad-PTEN wt expressing wild type PTEN, Ad-PTEN G129E expressing the lipid-phosphatase dead PTEN G129E mutant. (**a**) Immunoblotting analysis after adenovirus infection. Cell lysates were prepared 2 days after infection and probed for PTEN, phospho-Akt, and total Akt expression. (**b**) Cells were harvested after 24 h of treatment with TNF (10 ng/ml) and the extent of apoptosis was determined by quantitation of DNA fragmentation

inhibits death receptor-mediated apoptosis (Scaffidi *et al.*, 1998). To determine the contribution of the mitochondrial pathway in LNCaP cells undergoing apoptosis, we examined the effect of overexpressing Bcl-2 on sensitivity to apoptosis. LNCaP-Bcl-2 cells were completely resistant to apoptosis induced by PTEN expression plus TNF, anti-Fas antibody or staurosporine (Figure 7). Control experiments verified similar levels of PTEN expression and suppression of phospho-Akt in LNCaP-Neo and LNCaP-Bcl-2 cells (data not shown). These data, in agreement with previous results (Davies *et al.*, 1999), suggest that PTEN-mediated apoptosis depends on the mitochondrial pathway.

PTEN-mediated apoptosis involves a FADD-dependent pathway for both death receptor-mediated and drug-induced apoptosis

Upon activation by binding to ligands, death receptors such as TNF receptor and Fas recruit and activate caspase-8 through an adaptor protein FADD. Drug-induced apoptosis under certain conditions is also dependent on the presence of intact FADD-dependent signaling pathways (Micheau *et al.*, 1999; Tang *et al.*, 1999). To test the involvement of the FADD-dependent pathway, a dominant negative mutant of FADD (ΔFADD) lacking the N-terminal death effector domain was introduced via adenovirus (Chinnaiyan *et al.*, 1996). Co-infection with Ad-ΔFADD efficiently blocked death receptor-mediated apoptosis induced by PTEN and TNF (Figure 8). Ad-ΔFADD also blocked drug-induced apoptosis by PTEN and staurosporine as well as PTEN and mitoxantrone. Ad-ΔFADD also inhibited apoptosis induced by higher

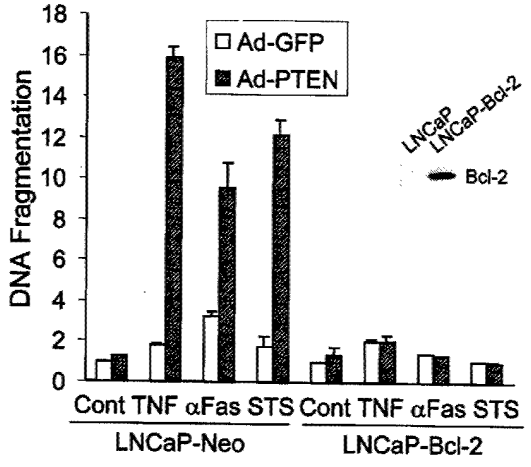


Figure 7 Bcl-2 blocks PTEN-mediated apoptosis. LNCaP-Neo vector control cells or LNCaP-Bcl-2 cells overexpressing Bcl-2 were infected with Ad-GFP or Ad-PTEN. The next day, cells were treated with TNF (20 ng/ml), or agonistic anti-Fas antibody IPO-4 (1 μg/ml) or staurosporine (0.1 μM), as indicated. After 24 h, the extent of apoptosis was determined. Insert, immunoblotting with anti-Bcl-2 antibody shows overexpression of Bcl-2 protein in LNCaP-Bcl-2 cells

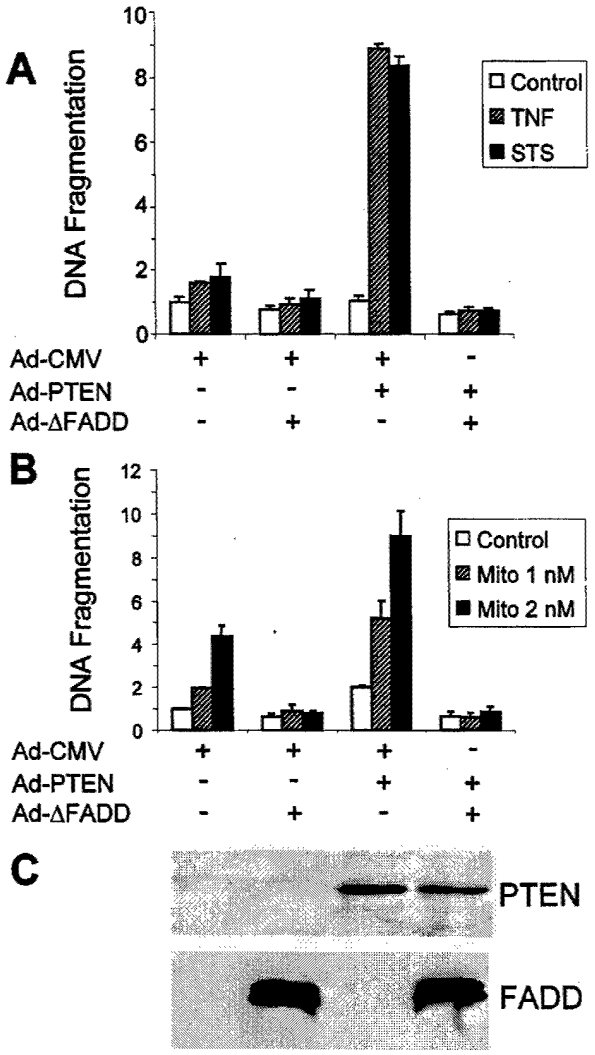


Figure 8 PTEN-mediated apoptosis involves a FADD-dependent pathway. (**a**) LNCaP cells were infected with control Ad-CMV virus (at m.o.i. of 20 or 10), or Ad-PTEN virus (at m.o.i. of 10), or Ad-ΔFADD virus (at m.o.i. of 10) or a combination as indicated. The total m.o.i. of each infection was kept constant at 20. Twenty-four hours after infection, the cells were treated with TNF (10 ng/ml) or agonistic anti-Fas antibody IPO-4 (1 μg/ml) or staurosporine (0.1 μM). After 24 h of treatment, the extent of apoptosis was determined. (**b**) LNCaP cells were infected with adenoviruses as above and were treated with mitoxantrone (1 or 2 nM) for 24 h as indicated. (**c**) Lysates from cells infected with adenoviruses as indicated above were immunoblotted to verify expression of PTEN and ΔFADD proteins. The ΔFADD protein appears as a doublet due to phosphorylation (Chinnaiyan *et al.*, 1996)

doses of staurosporine alone or mitoxantrone alone (data not shown and Figure 8b). To investigate the possibility of autocrine activation by death ligands in staurosporine-induced apoptosis, neutralizing antibodies against Fas and TNF-R1 were utilized. Pretreatment of cells with a neutralizing antibody against Fas (ZB4) or TNF-R1 (H398) had no effect on staurosporine-induced apoptosis (data not shown). In addition, quantitation of mRNA levels of components

of death receptor signaling such as Fas, Fas ligand, caspase-8, TNF-R1, TRADD, DR4, or DR5 by ribonuclease protection assays showed no difference between control and Ad-PTEN infected cells (data not shown). These data suggest that PTEN enhances drug-induced apoptosis through a ligand-independent but FADD-dependent signaling pathway.

PTEN facilitates BID cleavage and dominant negative FADD blocks BID cleavage

Death receptor signaling downstream of FADD involves recruitment and activation of caspase-8, which in turn cleaves BID. Cleaved BID subsequently activates the mitochondrial pathway by inducing the release of cytochrome *c* and activation of downstream caspases (Li *et al.*, 1998; Luo *et al.*, 1998). Since we have shown that PTEN plus staurosporine induces caspase-8 activation (Figure 5b), we hypothesized that PTEN-mediated, drug-induced apoptosis may also involve BID cleavage downstream of FADD and examined the status of BID in cells undergoing apoptosis in response to PTEN plus drug treatment. PTEN expression and treatment with a low dose of staurosporine or mitoxantrone led to BID cleavage and thereby loss of full length BID (Figure 9). BID cleavage and loss of full length BID did not occur with similar drug treatment in the absence of PTEN expression. Coexpression of dominant negative FADD completely blocked BID cleavage induced by PTEN plus drug treatment. Taken together, these data are consistent with the hypothesis that PTEN promotes drug-induced apoptosis by facilitating caspase-8 activation and BID cleavage through a FADD-dependent pathway.

Discussion

We show that PTEN sensitizes LNCaP prostate cancer cells to a broad range of apoptotic stimuli. PTEN enhances apoptosis induced by three different death receptors as well as by drugs and chemotherapeutic agents with distinctly different mechanisms of action. This is consistent with the hypothesis that PTEN may target a common cellular machinery used by many stimuli to execute the apoptotic program. However, not all apoptotic stimuli tested were affected by PTEN as PTEN did not sensitize cells to hyperosmotic shock or ultraviolet irradiation (data not shown).

PTEN regulates sensitivity to apoptosis by mechanisms involving the PI 3-kinase signaling pathway as the lipid phosphatase-deficient mutant of PTEN that retains protein phosphatase activity is unable to suppress activated Akt and is also unable to sensitize cells to apoptosis. Akt transduces antiapoptotic signals, in part, by phosphorylating and inactivating key proteins of the apoptotic machinery, such as BAD and caspase-9 (Cardone *et al.*, 1998; Datta *et al.*, 1997; del Peso *et al.*, 1997). Phosphorylated BAD is unable to heterodimerize with Bcl-2 and is no longer proapoptotic. PTEN would be expected to enhance mitochondrial signal amplification downstream of the death-inducing signaling complex. The inability of PTEN to sensitize Bcl-2 overexpressing cells to apoptosis is consistent with this model, as Bcl-2 overexpression results in inhibition of cytochrome *c* release from the mitochondria and blocks apoptosis in cells requiring mitochondrial amplification of signals downstream of the death-inducing signaling complex (Scaffidi *et al.*, 1998).

In addition to regulating the mitochondrial pathway, PTEN is likely to regulate sensitivity to apoptosis through other mechanisms. PTEN-mediated apoptosis is dependent on the adaptor protein FADD as the dominant negative FADD mutant (ΔFADD) blocks apoptosis initiated by PTEN plus TNF or staurosporine or mitoxantrone. Since this dominant negative FADD mutant uncouples downstream caspase activation from ligand-induced activation of death receptors, it is expected to inhibit apoptosis by PTEN plus TNF. However, the finding that dominant negative FADD also blocks apoptosis induced by PTEN plus staurosporine or mitoxantrone was unexpected since staurosporine and mitoxantrone have not been linked to the death receptor signaling pathway. Since neutralizing monoclonal antibodies against death receptors had no effect on staurosporine-induced apoptosis, these data raise a possibility that drug-induced apoptosis in LNCaP cells may involve a ligand-independent but FADD-dependent signaling pathway. A similar model has been postulated from work in other systems. Apoptosis induced by chemotherapeutic agents, nonsteroidal anti-inflammatory drugs, lipopolysaccharide and detachment from matrix can be blocked by expression of the dominant negative FADD mutant but not by neutralizing antibodies against death receptors (Choi *et al.*, 1998; Frisch, 1999; Han *et al.*, 2001; Micheau *et al.*, 1999; Rytomaa *et al.*, 1999). Previous reports have shown that chemotherapeutic agents cisplatin and camptothecin may induce ligand-independent aggregation of death receptors and recruitment of FADD to death receptors (Micheau *et*

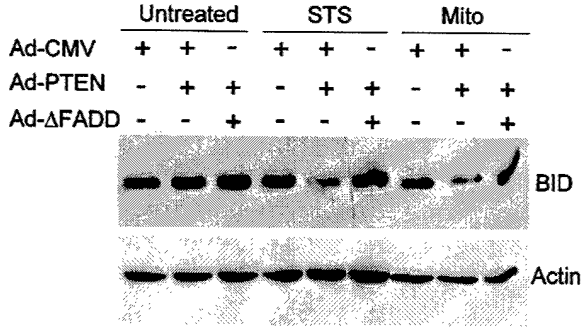


Figure 9 PTEN-mediated apoptosis proceeds through BID cleavage and dominant negative FADD blocks BID cleavage. LNCaP cells were infected with Ad-CMV or Ad-PTEN or Ad-ΔFADD and treated with staurosporine (0.1 μM) or mitoxantrone (2 nM) as detailed in Figure 8. Equal amounts of proteins were immunoblotted with anti-BID antibody to determine the level of full length 22-kDa BID. Immunoblotting with anti-actin antibody confirmed equivalent loading

al., 1999; Shao *et al.*, 2001). Alternately, FADD may operate completely independently of death receptors as there is accumulating evidence that FADD may be involved in other functions in addition to death receptor signaling. FADD-null mice are not viable and FADD may be required for proliferation of T cells (Newton *et al.*, 1998; Yeh *et al.*, 1998). More work is necessary to elucidate how PTEN affects the FADD-dependent apoptotic signaling pathway. However, one caveat in interpretation of experiments performed with overexpression of a dominant negative FADD mutant is that the FADD mutant may nonspecifically inhibit molecules other than FADD.

Death receptor-mediated apoptotic signaling proceeds through caspase-8 activation and BID cleavage downstream of FADD. Therefore, the status of caspase-8 and BID was examined in PTEN-mediated, drug-induced apoptosis. PTEN expression facilitates caspase-8 activation and BID cleavage in response to low doses of staurosporine and mitoxantrone (Figures 5b and 9). In the absence of PTEN, these low doses of drugs do not induce apoptosis or lead to caspase-8 activation or BID cleavage. Dominant negative FADD blocks BID cleavage induced by PTEN plus drug treatment, presumably by inhibiting caspase-8 activation. Cleaved BID induces oligomerization of proapoptotic BAK or BAX molecules on the mitochondrial membrane, triggering cytochrome *c* release (Eskes *et al.*, 2000; Wei *et al.*, 2000). A recent report shows that cells doubly deficient in BAX and BAK are protected from apoptosis induced by cleaved BID (Wei *et al.*, 2001). Furthermore, these BAX, BAK-deficient cells are resistant to apoptotic stimuli that produce mitochondrial damage, such as staurosporine and etoposide, suggesting that these agents require apoptotic signals upstream of mitochondria (Wei *et al.*, 2001). Our data implicating the FADD-dependent pathway that proceeds through BID cleavage in staurosporine- and mitoxantrone-induced apoptosis in the presence of PTEN is consistent with this idea. Taken together, these findings suggest the existence of an apoptotic signaling pathway from FADD to caspase-8 to BID to mitochondria in drug-induced apoptosis. PTEN expression lowers the threshold for drug-induced apoptosis by facilitating caspase-8 activation and BID cleavage downstream of FADD. These findings are consistent with two recent reports demonstrating that Akt protects LNCaP cells from apoptosis by inhibiting BID cleavage (Nesterov *et al.*, 2001; Thakkar *et al.*, 2001).

Another potential mechanism by which PTEN sensitizes to TNF- and chemotherapy-induced apoptosis is through inhibition of the inducible transcription factor NF-κB since NF-κB activation by TNF and chemotherapy plays a critical role in protecting cells from apoptosis (Wang *et al.*, 1996). NF-κB, normally sequestered in the cytoplasm in complex with IκB, translocates to the nucleus after signal-induced phosphorylation and degradation of IκB, and in the nucleus activates transcription of antiapoptotic target genes. PTEN does not appear to affect initial steps of NF-κB activation as there was no difference in nuclear translocation or induction of DNA binding activity of NF-κB after TNF treatment between control and PTEN-expressing LNCaP cells. Instead, PTEN inhibits the transactivation potential of the p65/RelA subunit of NF-κB in the nucleus and suppresses the transcription of certain target genes of NF-κB (M Mayo *et al.*, manuscript submitted). This is consistent with the postulated role of Akt in activating the transcriptional function of the p65/RelA subunit of NF-κB (Madrid *et al.*, 2000; Sizemore *et al.*, 1999). These findings suggest that NF-κB is a downstream target of PTEN and that PTEN inhibits the antiapoptotic function of NF-κB in sensitizing cells to TNF-induced apoptosis.

The effect of PTEN on TNF-induced apoptosis may be likened to cycloheximide, an agent that has widely been used to induce sensitivity to death ligands. Cycloheximide did not affect the activation status of Akt in LNCaP cells (data not shown). Cycloheximide is postulated to act by inhibiting translation of labile antiapoptotic proteins whose expression is induced by NF-κB. However, identity of these proteins in LNCaP cells has been elusive (Nesterov *et al.*, 2001). It is possible that PTEN and cycloheximide may ultimately have similar effects if PTEN inhibits transcription of NF-κB target genes and cycloheximide inhibits translation of newly transcribed genes. Further experiments are necessary to elucidate the target genes regulated by PTEN in inducing sensitivity to TNF.

Loss of PTEN is frequently associated with both localized and advanced prostate cancer specimens (McMenamin *et al.*, 1999; Suzuki *et al.*, 1998b; Whang *et al.*, 1998). In mouse models, inactivation of one of PTEN alleles leads to hyperplasia and dysplasia of prostate epithelial cells, eventually resulting in prostate cancer (Di Cristofano *et al.*, 1998; Podsypanina *et al.*, 1999; Suzuki *et al.*, 1998a). These findings point to loss of PTEN as one of key steps in prostate carcinogenesis. Data presented in this work show that PTEN plays a critical role in regulating the apoptotic threshold to multiple stimuli, including death ligands and chemotherapeutic agents. Therefore, reconstitution of PTEN function or inhibition of Akt represents a promising strategy for overcoming resistance of prostate cancer cells to chemotherapy-induced apoptosis.

Materials and methods

Materials and cell culture

TNF-α, staurosporine, mitoxantrone, and propidium iodide were obtained from Sigma (St Louis, MO, USA). Anti-Fas agonistic antibody IPO-4 and antagonistic antibody ZB4 were obtained from Kamiya Biomedical (Seattle, WA, USA). Anti-TNF-R1 antibody (H398) was obtained from Alexis (San Diego, CA, USA). z-VAD-fmk was obtained from Calbiochem (San Diego, CA, USA). LNCaP cells (American Tissue Type Collection, Manassas, VA, USA) were cultured in phenol red-free RPMI medium supplemented with 10% fetal calf serum. LNCaP cells overexpressing Bcl-2, previously described (Marcelli *et al.*, 1999; Raffo *et al.*, 1995), were kindly provided by Dr Marco Marcello.

Construction of adenovirus

For construction of a replication-defective recombinant adenovirus, Ad-PTEN, expressing PTEN from the CMV promoter, the PTEN cDNA was subcloned into the shuttle vector pACCMVpLpA (Gomez-Foix *et al.*, 1992) and cotransfected into 293 cells along with pJM17. The recombinant adenovirus was isolated by plaque purification. Ad-GFP virus expressing enhanced green fluorescence protein, constructed similarly, was provided by Dr Lily Wu (Department of Urology, University of California, LA, CA, USA). Another set of recombinant adenoviruses expressing the wildtype or the G129E mutant PTEN from the CMV promoter, Ad-PTEN wt and Ad-PTEN G129E, was constructed using the AdEasy system as described, after subcloning the PTEN wt and PTEN G129E fragments into the pShuttleCMV vector (He *et al.*, 1998). Ad-ΔFADD expressing the truncated dominant negative FADD protein was obtained from Dr David Brenner (Bradham *et al.*, 1998). Recombinant adenoviruses were purified through banding in a CsCl gradient by the UNC Viral Vector Laboratory.

Adenovirus infection and apoptosis assays

Preliminary experiments with Ad-GFP showed that the adenovirus dose of multiplicity of infection (m.o.i.) of 10 can infect 99.9% of LNCaP cells (data not shown). Therefore, we used m.o.i. of 10 in all subsequent experiments. LNCaP cells were plated at a density of 5×10^5 cells per well in a 6-well plate the day before infection. On day 0, cells were infected with recombinant adenovirus. On day 1, cells were treated with indicated agents. On day 2, after 24 h of treatment, both detached and adherent cells were harvested and processed for quantitation of DNA fragmentation using the Cell Death ELISA Plus kit (Roche, Indianapolis, IN, USA) according to the manufacturer's directions. For determination of DNA content by flow cytometry, cells were fixed in 80% ethanol overnight and stained with propidium iodide (50 μg/ml) and analysed using FACSCalibur (Becton Dickinson).

Caspase assay

Caspase-3 like activity in cell lysates was assayed using a commercially available kit (Caspase-3 Cellular Activity Assay Kit PLUS, Biomol, Plymouth Meeting, PA, USA). Briefly, 25 μg of protein was incubated with a colorimetric substrate DEVD-pNA and cleavage of the substrate was monitored by measuring optical density at 405 nm.

Immunoblotting

Cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter and probed with antibody as indicated. Goat anti-PTEN antibody (Santa Cruz, Santa Cruz, CA, USA) or mouse anti-PTEN monoclonal antibody (Cascade Bioscience, Winchester, MA, USA) was used to detect PTEN. Phospho-Akt antibody (Cell Signaling Technology, Beverly, MA, USA) detects Akt phosphorylated on Ser-473 whereas pan-Akt antibody (Cell Signaling Technology) detects both phosphorylated and unphosphorylated Akt. Anti-Bcl-2 monoclonal antibody (Santa Cruz), AU1 monoclonal antibody (Babco, Richmond, CA, USA), used to detect tagged ΔFADD, anti-BID antibody (Cell Signaling Technology) and anti-actin antibody (Sigma) were obtained commercially. Anti-caspase 8 monoclonal antibody (clone C15) (Scaffidi *et al.*, 1997) was kindly provided by Peter Krammer.

Acknowledgments

This work was supported by grants from the Department of Defense Prostate Cancer Research Program (DAMD17-00-1-0037) and NIH (CA85772). We thank Ralph Buttyan, Marco Marcelli, Lily Wu, David Brenner, Peter Krammer and Charles Sawyers for reagents, and Marty Mayo, Al Baldwin, Chris Gregory and Charles Sawyers for discussions and reviewing the manuscript.

References

Ashkenazi A and Dixit VM. (1999). *Curr. Opin. Cell. Biol.*, **11,** 255–260.

Bradham CA, Qian T, Streetz K, Trautwein C, Brenner DA and Lemasters JJ. (1998). *Mol. Cell. Biol.*, **18,** 6353–6364.

Budihardjo I, Oliver H, Lutter M, Luo X and Wang X. (1999). *Annu. Rev. Cell. Dev. Biol.*, **15,** 269–290.

Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S and Reed JC. (1998). *Science,* **282,** 1318–1321.

Chinnaiyan AM, Tepper CG, Seldin MF, O'Rourke K, Kischkel FC, Hellbardt S, Krammer PH, Peter ME and Dixit VM. (1996). *J. Biol. Chem.*, **271,** 4961–4965.

Choi KB, Wong F, Harlan JM, Chaudhary PM, Hood L and Karsan A. (1998). *J. Biol. Chem.*, **273,** 20185–20188.

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y and Greenberg ME. (1997). *Cell,* **91,** 231–241.

Davies MA, Koul D, Dhesi H, Berman R, McDonnell TJ, McConkey D, Yung WK and Steck PA. (1999). *Cancer Res.*, **59,** 2551–2556.

del Peso L, Gonzalez-Garcia M, Page C, Herrera R and Nunez G. (1997). *Science,* **278,** 687–689.

Di Cristofano A, Pesce B, Cordon-Cardo C and Pandolfi PP. (1998). *Nat. Genet.*, **19,** 348–355.

Eskes R, Desagher S, Antonsson B and Martinou JC. (2000). *Mol. Cell. Biol.*, **20,** 929–935.

Frisch SM. (1999). *Curr. Biol.*, **9,** 1047–1049.

Gomez-Foix AM, Coats WS, Baque S, Alam T, Gerard RD and Newgard CB. (1992). *J. Biol. Chem.*, **267,** 25129–25134.

Han Z, Pantazis P, Wyche JH, Kouttab N, Kidd VJ and Hendrickson EA. (2001). *J. Biol. Chem.*, **276,** 38748–38754.

He TC, Zhou S, da Costa LT, Yu J, Kinzler KW and Vogelstein B. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 2509–2514.

Hlobilkova A, Guldberg P, Thullberg M, Zeuthen J, Lukas J and Bartek J. (2000). *Exp. Cell. Res.*, **256,** 571–577.

Klein KA, Reiter RE, Redula J, Moradi H, Zhu XL, Brothman AR, Lamb DJ, Marcelli M, Belldegrun A, Witte ON and Sawyers CL. (1997). *Nat. Med.*, **3,** 402–408.

Kulik G, Carson JP, Vomastek T, Overman K, Gooch BD, Srinivasula S, Alnemri E, Nunez G and Weber MJ. (2001). *Cancer Res.*, **61,** 2713–2719.

Li H, Zhu H, Xu CJ and Yuan J. (1998). *Cell,* **94,** 491–501.

Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH and Parsons R. (1997a). *Science*, **275**, 1943–1947.

Li P, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES and Wang X. (1997b). *Cell*, **91**, 479–489.

Luo X, Budihardjo I, Zou H, Slaughter C and Wang X. (1998). *Cell*, **94**, 481–490.

Madrid LV, Wang CY, Guttridge DC, Schottelius AJ, Baldwin Jr AS and Mayo MW. (2000). *Mol. Cell. Biol.*, **20**, 1626–1638.

Maehama T and Dixon JE. (1998). *J. Biol. Chem.*, **273**, 13375–13378.

Maier D, Jones G, Li X, Schonthal AH, Gratzl O, Van Meir EG and Merlo A. (1999). *Cancer Res.*, **59**, 5479–5482.

Marcelli M, Cunningham GR, Walkup M, He Z, Sturgis L, Kagan C, Mannucci R, Nicoletti I, Teng B and Denner L. (1999). *Cancer Res.*, **59**, 382–390.

McMenamin ME, Soung P, Perera S, Kaplan I, Loda M and Sellers WR. (1999). *Cancer Res.*, **59**, 4291–4296.

Micheau O, Solary E, Hammann A and Dimanche-Boitrel MT. (1999). *J. Biol. Chem.*, **274**, 7987–7992.

Myers MP, Pass I, Batty IH, Van der Kaay J, Stolarov JP, Hemmings BA, Wigler MH, Downes CP and Tonks NK. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 13513–13518.

Myers MP, Stolarov JP, Eng C, Li J, Wang SI, Wigler MH, Parsons R and Tonks NK. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 9052–9057.

Nesterov A, Lu X, Johnson M, Miller GJ, Ivashchenko Y and Kraft AS. (2001). *J. Biol. Chem.*, **276**, 10767–10774.

Newton K, Harris AW, Bath ML, Smith KG and Strasser A. (1998). *EMBO J.*, **17**, 706–718.

Podsypanina K, Ellenson LH, Nemes A, Gu J, Tamura M, Yamada KM, Cordon-Cardo C, Catoretti G, Fisher PE and Parsons R. (1999). *Proc. Natl. Acad. Sci. USA*, **96**, 1563–1568.

Raffo AJ, Perlman H, Chen MW, Day ML, Streitman JS and Buttyan R. (1995). *Cancer Res.*, **55**, 4438–4445.

Rytomaa M, Martins LM and Downward J. (1999). *Curr. Biol.*, **9**, 1043–1046.

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH and Peter ME. (1998). *EMBO J.*, **17**, 1675–1687.

Scaffidi C, Medema JP, Krammer PH and Peter ME. (1997). *J. Biol. Chem.*, **272**, 26953–26958.

Shao RG, Cao CX, Nieves-Neira W, Dimanche-Boitrel MT, Solary E and Pommier Y. (2001). *Oncogene*, **20**, 1852–1859.

Sizemore N, Leung S and Stark GR. (1999). *Mol. Cell. Biol.*, **19**, 4798–4805.

Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP and Mak TW. (1998). *Cell*, **95**, 29–39.

Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DH and Tavtigian SV. (1997). *Nat. Genet.*, **15**, 356–362.

Suzuki A, de la Pompa JL, Stambolic V, Elia AJ, Sasaki T, del Barco Barrantes I, Ho A, Wakeham A, Itie A, Khoo W, Fukumoto M and Mak TW. (1998a). *Curr. Biol.*, **8**, 1169–1178.

Suzuki H, Freije D, Nusskern DR, Okami K, Cairns P, Sidransky D, Isaacs WB and Bova GS. (1998b). *Cancer Res.*, **58**, 204–209.

Tang D, Lahti JM, Grenet J and Kidd VJ. (1999). *J. Biol. Chem.*, **274**, 7245–7252.

Thakkar H, Chen X, Tyan F, Gim S, Robinson H, Lee C, Pandey SK, Nwokorie C, Onwudiwe N and Srivastava RK. (2001). *J. Biol. Chem.*, **276**, 38361–38369.

Wang CY, Mayo MW and Baldwin Jr AS. (1996). *Science*, **274**, 784–787.

Wei MC, Lindsten T, Mootha VK, Weiler S, Gross A, Ashiya M, Thompson CB and Korsmeyer SJ. (2000). *Genes Dev.*, **14**, 2060–2071.

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB and Korsmeyer SJ. (2001). *Science*, **292**, 727–730.

Weng LP, Brown JL and Eng C. (2001). *Hum. Mol. Genet.*, **10**, 599–604.

Whang YE, Wu X, Suzuki H, Reiter RE, Tran C, Vessella RL, Said JW, Isaacs WB and Sawyers CL. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 5246–5250.

Wu X, Senechal K, Neshat MS, Whang YE and Sawyers CL. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 15587–15591.

Yeh WC, Pompa JL, McCurrach ME, Shu HB, Elia AJ, Shahinian A, Ng M, Wakeham A, Khoo W, Mitchell K, El-Deiry WS, Lowe SW, Goeddel DV and Mak TW. (1998). *Science*, **279**, 1954–1958.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 277, No. 13, Issue of March 29, pp. 11116–11125, 2002
Printed in U.S.A.

PTEN Blocks Tumor Necrosis Factor-induced NF-κB-dependent Transcription by Inhibiting the Transactivation Potential of the p65 Subunit*

Received for publication, September 7, 2001, and in revised form, December 18, 2001
Published, JBC Papers in Press, January 17, 2002, DOI 10.1074/jbc.M108670200

Marty W. Mayo‡§¶, Lee V. Madrid‖, Sandy D. Westerheide‖, David R. Jones§, Xiu-Juan Yuan‖, Albert S. Baldwin, Jr.‖**‡‡, and Young E. Whang‖§§**

*From the ‡Department of Biochemistry and Molecular Genetics and the §Department of Surgery, the University of Virginia, Charlottesville, Virginia 22908 and the ‖Lineberger Comprehensive Cancer Center, **Curriculum in Genetics and Molecular Biology, the ‡‡Department of Biology and the §§Department of Medicine, University of North Carolina, Chapel Hill, North Carolina 27599*

PTEN is a lipid phosphatase responsible for down-regulating the phosphoinositide 3-kinase product phosphatidylinositol 3,4,5-triphosphate. Phosphatidylinositol 3,4,5-triphosphate is involved in the activation of the anti-apoptotic effector target, Akt. Although the Akt pathway has been implicated in regulating NF-κB activity, it is controversial as to whether Akt activates NF-κB predominantly through mechanisms that regulate nuclear translocation or transactivation potential. In this report, we utilized PTEN as a natural biological inhibitor of Akt activity to study the effects on tumor necrosis factor (TNF)-induced activation of NF-κB. We found that the reintroduction of PTEN into prostate cells inhibited TNF-stimulated NF-κB transcriptional activity. PTEN failed to block TNF-induced IKK activation, IκBα degradation, p105 processing, p65 (RelA) nuclear translocation, and DNA binding of NF-κB. However, PTEN inhibited NF-κB-dependent transcription by blocking the ability of TNF to stimulate the transactivation domain of the p65 subunit. PTEN also inhibited the transactivation potential of the cyclic AMP-response element-binding protein, but this was not observed for c-Jun. The transactivation potential of p65 following TNF stimulation could be rescued from PTEN-dependent repression by re-introducing expression constructs encoding activated forms of phosphoinositide 3-kinase, Akt, or Akt and IKK. The ability of PTEN to inhibit the TNF-induced transactivation function of p65 is important, because expression of PTEN blocked TNF-stimulated NF-κB-dependent gene expression, thus sensitizing cells to TNF-induced apoptosis. Maintenance of the PTEN tumor suppressor protein is therefore required to modulate Akt activity and to concomitantly control the transcriptional activity of the anti-apoptotic transcription factor NF-κB.

PTEN, also known as *MMAC1* or *TEP1*, is a tumor suppressor gene inactivated in many common malignancies, including glioblastoma, melanoma, endometrial, lung, and prostate cancer (1–5). The genetic evidence that PTEN is an important tumor suppressor protein is supported by the fact that heterozygous disruption of the *PTEN* gene in knockout mice results in the spontaneous development of tumors late in life (6). PTEN has been implicated in regulating cell survival signaling through the phosphatidylinositol 3-kinase (PI3K)[1]/Akt pathway. PTEN dephosphorylates the D3 position of the key lipid second messenger phosphatidylinositol 3,4,5-triphosphate (PIP_3) (6–8). PIP_3, produced by PI3K following activation by receptor tyrosine kinases, activated Ras, or G proteins, leads to the stimulation of several downstream targets, including the serine/threonine protein kinase Akt (also known as protein kinase B) (1–5). Activated Akt protects cells from apoptotic death by phosphorylating substrates such as BAD, procaspase-9, and forkhead transcription family members (9–11). Akt has also been shown to prolong cell survival by delaying p53-dependent apoptosis (12). Recently, Akt has been proposed to regulate permeability transition pore opening within the mitochondrial membrane by increasing the coupling of glucose metabolism to oxidative phosphorylation (13). Finally, multiple laboratories have demonstrated that the PI3K/Akt pathway provides cell survival signals, in part, through the activation of the NF-κB transcription factor (14–17).

NF-κB, classically a heterodimer composed of the p50 and p65 subunits, is a transcription factor whose activity is tightly regulated at multiple levels (18–21). NF-κB is normally sequestered in the cytoplasm as an inactive complex bound by an inhibitor known as IκB (18). Following cellular stimulation, IκB proteins become phosphorylated by the IκB kinase (IKK), which subsequently targets IκB for ubiquitination and degradation through the 26 S proteasome (20). The degradation of IκB proteins liberates NF-κB, allowing this transcription factor to translocate to the nucleus. In addition to regulation by IκB, NF-κB is also regulated by phosphorylation events that positively up-regulate the transactivation potential of NF-κB subunits (22). The transactivation domains of NF-κB have been

* This work was supported by National Institutes of Health Grants CA78595 (to M. W. M.), CA72771 (to A. S. B.), CA85772 (to Y. E. W.), CA83920 (to D. R. J.), and CA75080 (to A. S. B. and M. W. M.), by the University of Virginia Paul Mellon Prostate Cancer Research Institute (to M. W. M.), and by the United States Army Prostate Cancer Research Program Grant DAMD17-00-1-0037 (to Y. E. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Genetics, Box 800733, University of Virginia, Charlottesville, VA 22908. Tel.: 434-924-2509; Fax: 434-924-5069; E-mail: mwm3y@virginia.edu.

[1] The abbreviations used are: PI3K, phosphoinositide 3-kinase; PIP_3, phosphatidylinositol 3,4,5-triphosphate; IKK, IκB kinase; TNF, tumor necrosis factor; CREB, cyclic AMP-response element-binding protein; IL, interleukin; MHC, major histocompatibility complex; GFP, green fluorescent protein; Ad, adenovirus; EMSAs, electrophoretic mobility shift assays; pfu, plaque-forming units; CaMKK, calmodulin-dependent kinase kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay.

shown to be regulated by the catalytic domain of protein kinase A, casein kinase II, and by IKK itself (23–27). Although signals that regulate nuclear translocation of NF-κB have been regarded as the primary mechanism of NF-κB activation, alternative mechanisms involving the transactivation potential of p65 have been shown to be critical for NF-κB activation *in vitro* and *in vivo* (22, 28, 29).

Several different laboratories, including our own, have shown that various growth factors, cytokines, and oncogenes require PI3K- and Akt-dependent pathways for full NF-κB activation (30–36). Despite this consensus, the exact mechanism by which Akt pathways activate NF-κB remains controversial (37). Activation of the Akt pathway has been reported to stimulate IKK-dependent IκB degradation and nuclear translocation of NF-κB (14, 31). Other reports including our own (15, 30, 36) have shown that Akt-dependent activation of NF-κB occurs predominantly by stimulating the transactivation potential of the p65 subunit, rather than inducing signals that result in NF-κB nuclear translocation via IκB degradation. Recently, two reports (38, 39) from independent laboratories analyzed the effects of PTEN-dependent inhibition of the PI3K and Akt pathway on cytokine-induced activation of NF-κB. Both reports concluded that PTEN expression blocked IL-1β or TNF-induced NF-κB activation; however, major discrepancies exist between these two studies. Koul *et al.* (38) showed that PTEN-dependent inhibition of Akt failed to block IL-1β-induced IκB degradation and nuclear translocation of p65 but rather inhibited NF-κB-DNA binding. In this study, it was proposed that PTEN functioned to inhibit phosphorylation of the p50 subunit of NF-κB, thus inhibiting the DNA binding potential of NF-κB (38). In contrast, Gustin *et al.* (39) reported that PTEN inhibited NF-κB transcriptional activity by impairing TNF-induced activation of Akt and the IKK complex, suggesting that the PTEN-mediated inhibition of IKK activity blocked the TNF-induced nuclear translocation and DNA binding potential of NF-κB. Although both of these studies (38, 39) demonstrate that PTEN is capable of inhibiting cytokine-induced activation of NF-κB, it remains ambiguous as to whether PTEN-dependent inhibition of Akt inhibits NF-κB by down-regulating signals that control nuclear translocation, DNA binding, and/or transactivation potential of NF-κB.

To address the involvement of PI3K and Akt in TNF-induced activation of NF-κB, we utilized PTEN-deficient prostate cell lines that constitutively express activated Akt because of a loss of PTEN lipid phosphatase activity (40, 41). In this study, we demonstrate that re-introduction of PTEN into prostate cells results in a down-regulation of Akt activity and a loss of TNF-induced NF-κB-dependent transcription without blocking IKK-induced IκB degradation, p105 processing, p65 nuclear translocation, or NF-κB DNA binding activity. However, we find that neither Akt nor IKK is dispensable for TNF to stimulate the transactivation potential of the p65 subunit of NF-κB in prostate epithelial cells. We demonstrate that PTEN elicits selective inhibition by blocking signal transduction pathways that are responsible for targeting the transactivation potential of NF-κB and CREB but not for c-Jun. The ability of PTEN to inhibit Akt activity and subsequently block TNF-induced transcriptional activity of NF-κB led to abrogation of the anti-apoptotic function of NF-κB. This work demonstrates that re-introduction of PTEN sensitizes prostate epithelial cells to TNF-induced apoptosis, in part by down-regulating the transactivation potential of NF-κB. Thus, NF-κB is a relevant target of the tumor suppressor function of PTEN.

MATERIALS AND METHODS

Cell Culture, Reagents, and Plasmid Constructs—Human prostate cancer cells, LNCaP, were grown in T-media (Invitrogen) supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, UT) and penicillin/streptomycin. PC-3 and DU-145 cells were cultured in Dulbecco's modified Eagle's medium-H (Invitrogen) supplemented with 10% fetal bovine serum and penicillin/streptomycin. The 3×-κB luciferase (3×-κB-Luc) reporter construct contains four NF-κB DNA-binding consensus sites originally identified in the MHC class I promoter, fused upstream to firefly luciferase (42). The Gal-4 luciferase construct (Gal4-Luc) contains four Gal-4 DNA-binding consensus sites, derived from the yeast *GAL-4* gene promoter, cloned upstream of luciferase cDNA (42). Plasmids encoding the Gal4-p65 fusion protein have the yeast Gal-4 DNA binding domain fused to the transactivation domain 1 (TA1) of NF-κB (43). Gal4-cJun-(1–223) and Gal4-CREB-(1–283) were commercially purchased (Stratagene, La Jolla, CA). His-tagged β-galactosidase encoding plasmid pCMV-LacZ was obtained from Invitrogen. Expression plasmids encoding constitutively activated Ha-Ras(V12), PI3K*, myristoylated Akt (M-Akt), and wild-type IKKβ (wtIKKβ) and plasmids encoding dominant negative PI3K (p85), Akt(K-M), and IKKβ(SS ≫ AA) proteins have been described previously (15). Plasmids encoding constitutively active FLAG-tagged CaMKKc were described previously (44). The expression vectors pcDNA3-PTENwt and pcDNA3-PTEN C124S have been described previously (41). The PTEN G129E mutant was constructed using the Quikchange site-directed mutagenesis kit (Stratagene) and verified by DNA sequence analysis. Recombinant human TNF was obtained from Promega (Madison, WI). PTEN (N19), His epitope tag (G-18), IκBα (C-21), p50 (N19), and Gal-4 (N19) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Pan-Ras antibody (Ab-4) was purchased from Calbiochem. Phospho-Akt-specific (Ser-473), pan-Akt, and IKKγ antibodies were obtained from New England Biolabs (Beverly, MA). p65-specific antibody was obtained through Rockland (Gilbertsville, PA), and M2 FLAG epitope tag and α-tubulin (T9026) were obtained from Sigma. The proteasome inhibitor MG132 (C2211) was obtained from Sigma.

Transfection and Luciferase Reporter Assays—LNCaP cells at 60–80% confluency were transiently transfected using Superfect reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Briefly, plasmid constructs (1 μg of DNA total) were diluted in serum-free media and mixed with the Superfect reagent. Complexes were allowed to form for 10 min before serum-containing media were added to the mixture. The cells were washed once with 1× phosphate-buffered saline, and Superfect-DNA complexes were added to the cells and placed in a humidified incubator at 37 °C with 5% CO_2. Three hours following the start of transfection, cells were washed with 1× phosphate-buffered saline and replenished with fresh serum-containing media. Twenty four hours post-transfection, cells were washed once with 1× phosphate-buffered saline and lysed in 0.25 M Tris-HCl (pH 7.4) following three freeze-thaws in a dry-ice/ETOH bath. Extracts were collected and cleared by centrifugation at 14,000 rpm. Protein concentrations were determined with the Bio-Rad protein assay dye reagent. Luciferase assays were performed on equal amounts of protein (100 μg/sample). D-Luciferin was used as a substrate, and relative light units were measured using an AutoLumat LB953 luminometer (Berthold Analytical Instruments). For control purposes, all cell groups that received PTEN were also co-transfected with pCMV-LacZ and assayed for transfection efficiency by counting β-galactosidase-positive cells as described previously (45).

Adenovirus Construction and Infection—Ad-PTEN is a replication-defective E1-deleted adenovirus expressing PTEN under the control of the cytomegalovirus promoter. Recombinant PTEN adenovirus was constructed as described previously (46). Recombinant virus was plaque-purified three times, and the structure was verified by restriction mapping of Hirt supernatant DNA. Ad-GFP, similarly constructed, expresses enhanced green fluorescent protein and was provided by Dr. Lily Wu (UCLA). Adenovirus was amplified in 293 cells and purified by banding in a cesium chloride density gradient.

Electrophoretic Mobility Shift Assays and Western Blot Analysis—Preparation of nuclear and cytoplasmic extracts and electrophoretic mobility shift assays (EMSAs) were performed as described previously (42). Briefly, nuclear extracts were prepared at the indicated times and incubated with [^{32}P]dCTP-labeled, double-stranded probe containing an NF-κB consensus site from the class I major histocompatibility complex (MHC) promoter. Labeled probe-nuclear complexes were incubated for 10 min at room temperature and separated on a 5% polyacrylamide gel. Subsequently, the gel was dried and exposed to x-ray film. Western blot analysis was performed by either analyzing 0.25 M Tris-HCl-lysed cell extracts or cytoplasmic and nuclear proteins on a 10% SDS-polyacrylamide gel. Total protein (50 μg) was separated by SDS-PAGE and transferred to nitrocellulose membranes. The indicated primary antibodies were incubated for 30 min, washed, and visualized by incubation

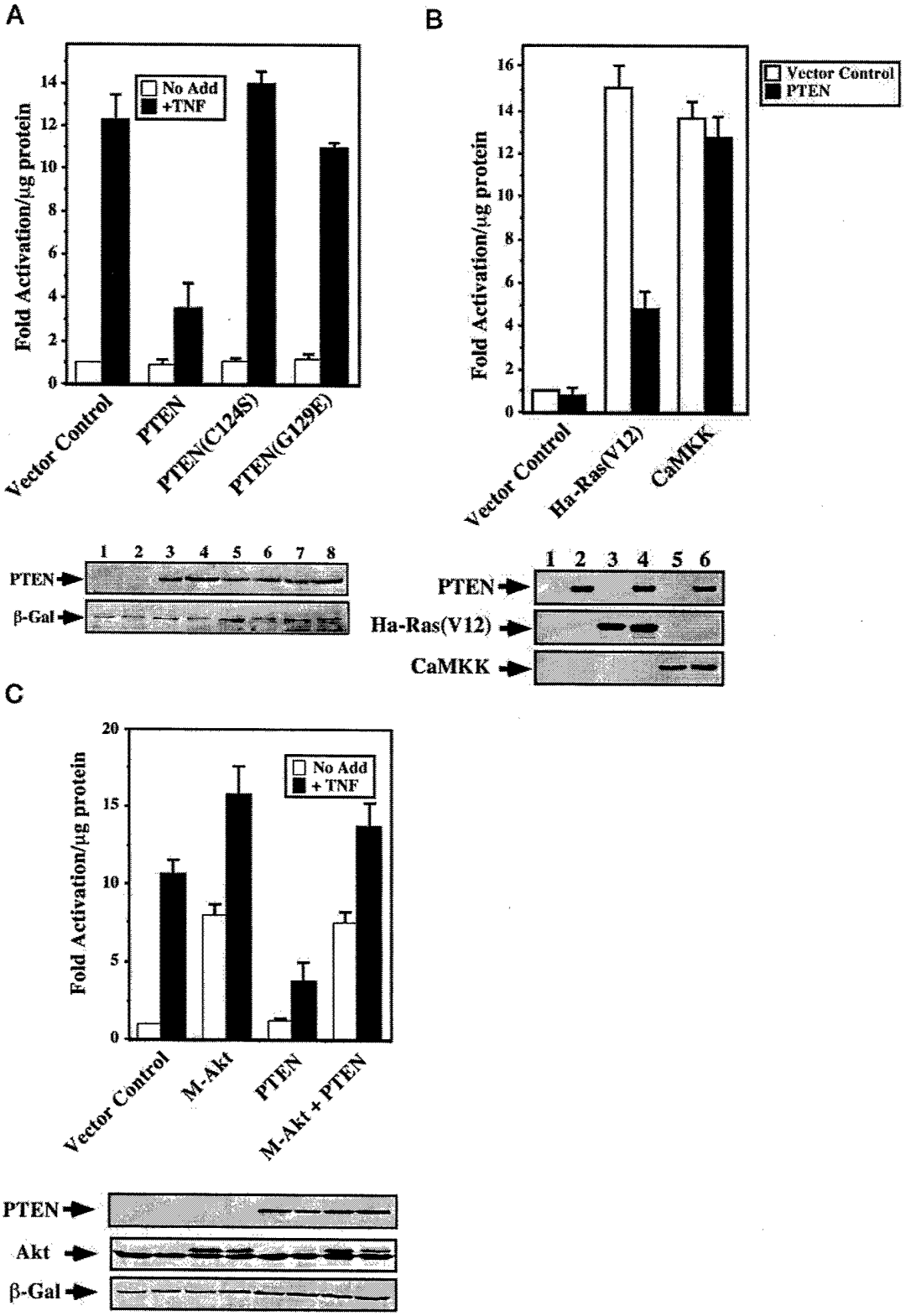


FIG. 1. **PTEN inhibits NF-κB-dependent transcription in response to TNF.** *A*, the lipid phosphatase activity of PTEN is required to suppress TNF-induced activation of NF-κB. LNCaP cells were transiently co-transfected with an NF-κB-responsive reporter (3×-κB-Luc, 0.5 μg) and the control plasmid (pCMV-LacZ) encoding the β-galactosidase enzyme. In addition, cells were also co-transfected with expression plasmids encoding wild-type PTEN, mutant PTEN(C124S), PTEN(G129E), or empty vector control (1 μg each). Eighteen hours following transfection, LNCaP cells were stimulated with TNF (10 ng/ml). Cell lysates were harvested 12 h post-TNF stimulation, and luciferase activity was assayed. Data are presented as fold activation, where the values obtained for the vector control group were normalized to 1. Results represent the mean ± S.D. of three independent experiments performed in triplicate. *Bottom panel*, total protein was isolated from a representative transfection experiment, and immunoblot analysis was performed for PTEN and His-tagged β-galactosidase (*β-Gal*) as described under "Materials and Methods." *B*, PTEN inhibits Ha-Ras(V12)-induced activation of NF-κB. LNCaP cells were co-transfected with 3×-κB-Luc (0.5 μg) and with empty vector control plasmid or expression constructs encoding constitutively active Ha-Ras(V12) or CaMKK (1 μg each). In addition, cells were also co-transfected with either empty vector control or with wild-type PTEN (1 μg each). Luciferase activities were determined 24 h following transfection. Data represent three individual experiments performed in triplicate. Western blot analysis confirmed the expression of PTEN,

with horseradish peroxidase-conjugated secondary antibodies and ECL chemiluminescent reagents (Amersham Biosciences).

IKK Immunokinase Assay—Subconfluent LNCaP cells infected with either Ad-GFP or Ad-PTEN for 48 h were either left untreated or were stimulated with 10 ng/ml TNF for 15 min. Whole cells extracts were immunoprecipitated with an antibody against IKKγ (New England Biolabs), and the immunoprecipitates were subject to an IKK assay (26) using GST-IκBα-(1–54) (4 μg) as a substrate. Samples were resolved on SDS-PAGE gels, dried, and subjected to autoradiography. Immunoprecipitated protein complexes were also analyzed by Western blot analysis to confirm that equal amounts of IKKγ had been analyzed during the IKK assay. Whole cell lysates were analyzed by Western blot analysis for PTEN expression.

Northern Blot Analysis—Logarithmically growing LNCaP cells were infected with either Ad-GFP or Ad-PTEN virus. Twenty four hours later, cells were either left untreated (t_0) or stimulated with TNF (10 ng/ml). Total RNAs were isolated using Trizol reagent (Invitrogen). RNAs (15 μg/lane) were resolved on a denaturing 1.8% agarose-formaldehyde gel, transferred to Hybond membrane (PerkinElmer Life Sciences), and crossed-linked. Gene expressions were determined by analyzing Northern blots with ^{32}P-labeled random probes generated from *NF-κB1*, *bcl-3*, or *GAPDH* cDNAs, and blots were hybridized with radiolabeled probes in Quickhyb (Stratagene). After a 2-h hybridization, the blots were washed twice in 2× SSC, 0.1% SDS for 15 min at room temperature and twice in 0.1× SSC, 0.1% SDS for 15 min at 60 °C. Northern blots were analyzed by autoradiography.

Apoptosis Assay—LNCaP cells were plated at 5 × 10^5 cells per well in a 6-well plate on day 0 and infected with adenovirus at 10 pfu/cell on day 1. TNF was added to the media on day 2. After 24 h of incubation with TNF, cells were harvested, and the extent of apoptosis was determined by quantitation of nucleosomes released into the cytoplasm using the Cell Death Detection ELISA Plus kit (Roche Molecular Biochemicals) according to the manufacturer's directions.

RESULTS

The Transcriptional Activity of NF-κB Is Inhibited by PTEN—Our laboratory has demonstrated previously (15, 36) that PI3K and Akt activate NF-κB predominantly by targeting the transactivation potential of the p65 subunit. Because PTEN is the predominant negative regulator of Akt *in vitro* and *in vivo* (1–6), we were interested in determining whether the PTEN tumor suppressor gene product could inhibit NF-κB transcriptional activity. To address this question we used the human prostate cell line LNCaP, in which endogenous Akt is constitutively active due to inactivation of PTEN (40, 41). LNCaP cells were transiently co-transfected with the NF-κB-responsive reporter 3×-κB-Luc and with wild-type PTEN or various PTEN mutants. Functionally inactive mutants included PTEN(C124S), which is defective in both protein and lipid phosphatase activity, and PTEN(G129E), which is selectively deficient in lipid phosphatase activity (7, 47). Following transfection, LNCaP cells were subsequently treated with TNF for 12 h, after which cell extracts were harvested and luciferase activities were analyzed. Cells transfected with an expression plasmid encoding wild-type PTEN displayed a reduction in TNF-induced NF-κB-dependent transcription, as compared with cells transfected with the empty vector control (Fig. 1*A*). The decrease in 3×-κB luciferase reporter activities observed following the expression of PTEN was not due to cell death, because an internal β-galactosidase reporter displayed similar levels of protein expression 12 h post-TNF addition (Fig. 1*A*). Importantly, the ability of PTEN to suppress NF-κB-dependent transcriptional activity was associated with the lipid phosphatase activity of this tumor suppressor gene product, because both PTEN(C124S) and PTEN(G129E) mutants were unable to block effectively the TNF-induced NF-κB transcriptional activity (Fig. 1*A*). In conclusion, re-introduction of PTEN into LNCaP cells did not block basal NF-κB activity but significantly inhibited TNF-induced NF-κB transcription (Fig. 1*A*). These results indicate that constitutive Akt activity alone, due to a loss of PTEN expression, was not enough to stimulate NF-κB activity and indicate that cellular stimulation is required for full NF-κB activation, which can be blocked by PTEN expression.

To determine whether the inhibition of NF-κB-dependent transcription by PTEN was specific to the ability of PTEN to block PI3K-dependent stimulation of Akt, additional transient reporter gene assays were performed. LNCaP cells were transiently co-transfected with activated forms of Ha-Ras or calmodulin-dependent kinase kinase (CaMKK) in the presence of either PTEN or vector control plasmid. Expression of activated Ha-Ras(V12) in LNCaP cells effectively up-regulated the NF-κB-responsive reporter, which was blocked by co-expression of PTEN protein (Fig. 1*B*). The expression of CaMKK, which is known to directly activate Akt in a PIP_3-independent manner (48), induced the transcriptional activity of NF-κB. NF-κB activation by CaMKK was not significantly blocked by PTEN (Fig. 1*B*). These results indicate that PTEN is capable of inhibiting both TNF- and Ras(V12)-induced transcriptional activation of NF-κB, presumably through its ability to down-regulate PI3K-induced PIP_3 levels.

To address further whether the ability of PTEN to block TNF-induced NF-κB transcription was associated with a down-regulation of Akt activity, LNCaP cells were transfected with a plasmid encoding a constitutively active Akt protein (M-Akt). Due to a myristoylation motif, the M-Akt protein constitutively inserts into the cytoplasmic membrane and no longer requires PIP_3 products generated by PI3K for kinase activity. Thus, we would predict that M-Akt would bypass PTEN-mediated effects and would allow TNF-dependent signals to activate NF-κB even in the presence of PTEN expression. As shown in Fig. 1*C*, expression of M-Akt in LNCaP cells stimulated the transcriptional activity of NF-κB. These results suggest that the overexpression of M-Akt acts to stimulate NF-κB through mechanisms different from endogenous Akt activity normally displayed in LNCaP cells. Regardless, NF-κB activity was increased further in M-Akt-transfected cells following stimulation with TNF (Fig. 1*C*). Consistent with the data presented in Fig. 1*A*, PTEN blocked TNF-induced activation of NF-κB. However, expression of M-Akt rescued PTEN-mediated suppression of NF-κB following TNF addition (Fig. 1*C*). The ability of M-Akt to overcome PTEN-mediated effects on TNF-dependent activation of NF-κB was not due to disproportionate transgene expression, because Western blot analysis demonstrated appropriate protein expression of PTEN, Akt, and the β-galactosidase control in co-transfection experiments (Fig. 1*C*). Therefore, expression of a constitutively active Akt mutant overcomes the ability of PTEN to block TNF-induced activation of NF-κB.

PTEN Inhibits NFκB through Mechanisms Independent of IκBα Degradation, p105 Processing, and p65 Nuclear Translocation—To elucidate the molecular mechanisms by which PTEN suppresses NF-κB-dependent transcription, LNCaP cells were infected with adenovirus directing the expression of either PTEN (Ad-PTEN) or green fluorescent protein (Ad-

HA-tagged Ha-Ras(V12), and FLAG-tagged CaMKK proteins. *C*, constitutively active Akt rescues TNF-induced activation of NF-κB following the expression of PTEN. LNCaP cells were co-transfected with 3×-κB-Luc (0.5 μg) and with empty vector control plasmid or expression constructs encoding constitutively active Akt (*M-Akt*), wild-type PTEN, or M-Akt and PTEN (1 μg each). Eighteen hours following transfections, cells were stimulated with TNF (10 ng/ml). Cells were harvested 12 h following the addition of TNF, and luciferase activities were analyzed. Results represent the mean ± S.D. of three independent experiments performed in triplicate. *Bottom panel*, Western blot analysis demonstrating expression of HA-tagged M-Akt, PTEN, and His-tagged β-galactosidase.

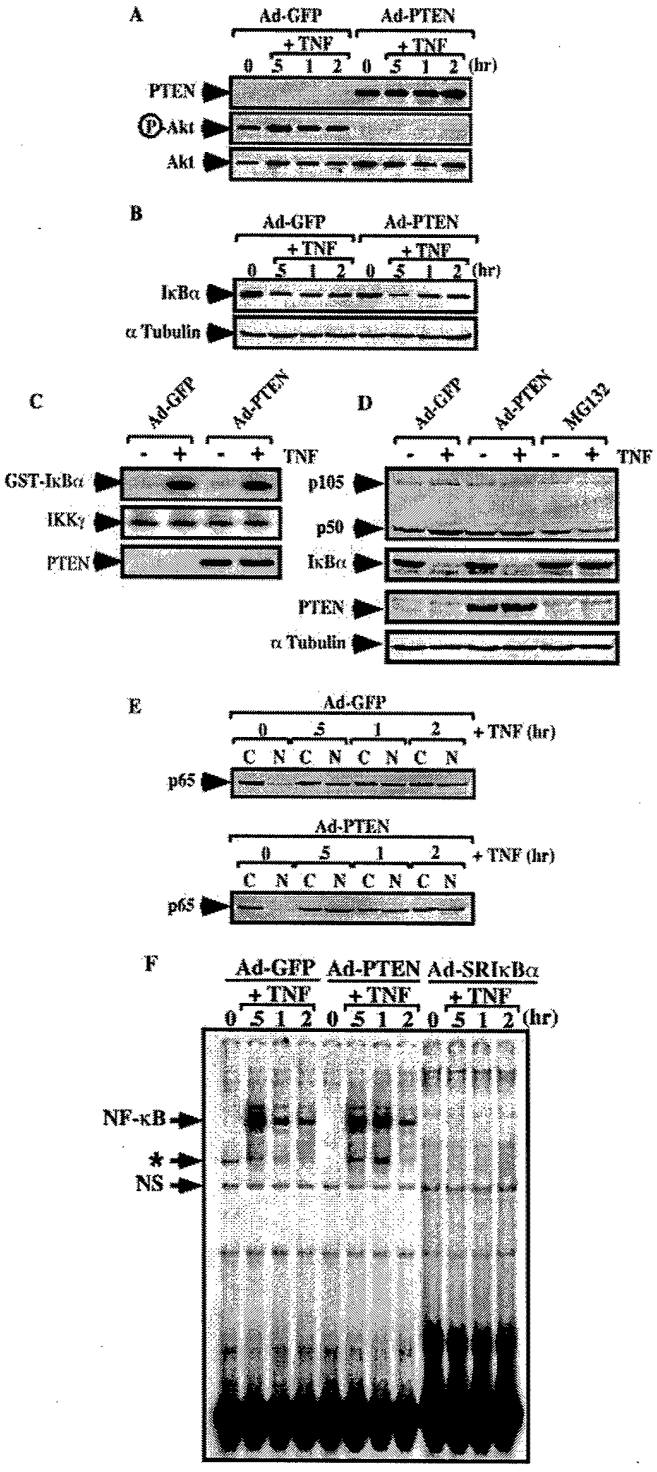


FIG. 2. **TNF stimulates IKK-dependent IκBα degradation, p65 nuclear translocation, p105 processing, and NF-κB DNA binding activity in LNCaP cells despite PTEN expression.** *A*, ectopic expression of PTEN efficiently down-regulates constitutively active Akt in LNCaP cells. LNCaP cells were infected with adenovirus encoding either green fluorescent protein (*Ad-GFP*) or PTEN (*Ad-PTEN*) at 10 pfu/cell. Twenty four hours following infection, cells were stimulated with TNF (10 ng/ml) over the time course indicated, and nuclear and cytoplasmic proteins were harvested from cells. Western blot analysis was performed using cytoplasmic extracts, and ectopic expression of PTEN was detected. Endogenous Akt activity was assessed using the phosphospecific antibody that detects phospho-Ser-473 on Akt. Total Akt protein levels were detected using a pan-specific antibody. *B–E*, PTEN expression in LNCaP cells fails to inhibit IKK activity, TNF-induced degradation of IκBα, p105 processing, and nuclear translocation of the p65 protein. *B*, cytoplasmic extracts (25 μg/lane) from Ad-GFP- and Ad-PTEN-infected LNCaP cells were analyzed for IκBα protein levels following TNF stimulation. Total IκBα and α-tubulin protein levels were detected. *C*, TNF-stimulated LNCaP cells expressing either GFP or PTEN were analyzed for IKK activity. Whole cell lysates (100 μg) were immunoprecipitated with anti-IKKγ and incubated with GST-IκBα-(1–54) in the presence of [γ-^{32}P]ATP. Immunoprecipitates and whole cell extracts were analyzed for IKKγ and PTEN expression, respectively, by Western blot analysis. *D*, p50 activity was measured as a function of p105 processing in TNF-stimulated LNCaP cells expressing either GFP or PTEN. LNCaP cells were infected with adenovirus, as described above, or treated with the proteasome inhibitor MG132 (20 μM) 1 h prior to the addition of TNF for 30 min. Total proteins (50 μg/lane) were subjected to SDS-PAGE, and Western blots were analyzed for protein expression. *E*, cytoplasmic (*C*) and nuclear (*N*) extracts were analyzed for TNF-induced nuclear translocation of the p65 subunit of NF-κB. *F*, PTEN fails to block TNF-induced DNA binding of NF-κB. Nuclear proteins (8 μg/reaction) were incubated with a ^{32}P-labeled double-stranded oligonucleotide corresponding to the NF-κB consensus site located in the MHC class I promoter. DNA-protein complexes were resolved on a non-denaturing polyacrylamide gel. The NF-κB-specific complex (composed of p65 and p50) is indicated by an *arrow*, as is a nonspecific (*NS*) band, which indicates relatively equal amounts of nuclear extract in each reaction. An *asterisk* indicates the p50 homodimer. Nuclear extracts isolated from LNCaP cells infected with the Ad-SRIκBα and then treated with TNF served as a positive control.

GFP). Adenoviral-mediated gene transfer is extremely efficient in these cells where, at 10 plaque-forming units (pfu) of virus per cell, 100% of cells display transgene expression (data not shown). Nuclear and cytoplasmic extracts were isolated from adenoviral infected cells following the addition of TNF over the time course indicated. As shown in Fig. 2*A*, cytoplasmic proteins isolated from LNCaP cells infected with Ad-PTEN demonstrated PTEN protein expression, as compared with Ad-GFP-infected control cells. Importantly, ectopic expression of the PTEN protein was functional in LNCaP cells, because lipid phosphatase activity of this tumor suppressor protein significantly down-regulated the level of activated phospho-specific Akt protein (Fig. 2*A*). The inability to detect phosphorylated Akt in Ad-PTEN-infected cells was not due to differences in protein loading, because similar levels of total Akt protein were detected when blots were re-analyzed using a pan-Akt antibody (Fig. 2*A*).

To elucidate whether PTEN expression blocks TNF-induced phosphorylation and proteasome-dependent degradation of IκB, cytoplasmic extracts were analyzed for the presence of IκBα protein. As shown in Fig. 2*B*, TNF-stimulated extracts displayed a loss of IκBα protein with similar kinetics in both Ad-GFP- and Ad-PTEN-infected LNCaP cells. Moreover, analysis of the p65 subunit of NF-κB confirmed that the addition of TNF led to an increase in nuclear accumulation regardless of whether the cells overexpressed the PTEN tumor suppressor gene product (Fig. 2*E*). Collectively, these results indicate that PTEN did not inhibit the transcriptional activity of NF-κB through a mechanism that blocked IκBα degradation and nuclear translocation of p65 in LNCaP cells.

Because Akt has been reported to be required for TNF-induced IKK activity (31, 39), LNCaP cells expressing PTEN were analyzed for IKK activity following TNF stimulation. As shown in Fig. 2*C*, LNCaP cells expressing PTEN displayed similar TNF-induced IKK activity, as compared with control cells. These results indicate that PTEN expression did not block TNF-induced IKK activity in these cells. Moreover, although LNCaP cells express constitutively active Akt, these cells fail to display constitutive IKK activity.

PTEN has also been reported to negatively regulate NF-κB through mechanisms affecting p50 activity (38). Because p50 activity is regulated predominantly through IKK-dependent phosphorylation and proteolysis of the p105 precursor protein (49–51), we analyzed whether PTEN blocked p50 activity by

inhibiting p105 processing in LNCaP cells following TNF stimulation. LNCaP cells did not display significant increases in p105 processing following the addition of TNF (Fig. 2*D*). Moreover, p50 levels did not change following the expression of PTEN or following the addition of the proteasome inhibitor MG132 (Fig. 2*D*). Consistent with Fig. 2*B*, PTEN also did not block TNF-induced degradation of IκBα, but degradation was blocked by pretreatment with MG132 (Fig. 2*D*). Therefore, in contrast to previous reports (38), PTEN failed to block TNF-induced NF-κB activity through mechanisms involving p50 activation. Collectively, these results are consistent with our findings that PTEN expression in LNCaP cells does not block IKK-dependent IκBα degradation or p105 processing nor does it affect p65 nuclear translocation following TNF stimulation (Fig. 2, *B*, *D*, and *E*).

To elucidate whether PTEN expression affected TNF-induced activation of NF-κB through mechanisms involving DNA binding, nuclear extracts were analyzed in EMSAs. As shown in Fig. 2*F*, adenoviral infected LNCaP cells expressing PTEN displayed similar NF-κB DNA binding profiles following the addition of TNF, as compared with cells receiving the Ad-GFP control virus. Interestingly, nuclear extracts from LNCaP cells infected with Ad-PTEN displayed slightly elevated DNA binding activity of NF-κB at 1 h post-TNF addition, as compared with Ad-GFP-infected cells (Fig. 2*F*). As a control, nuclear extracts isolated from LNCaP cells infected with Ad-SRIκBα, encoding a mutated form of IκBα that cannot be degraded following TNF stimulation, failed to display TNF-induced NF-κB activity (Fig. 2*F*). Similar amounts of nuclear extracts were analyzed in EMSAs, because the detection of a nonspecific band displayed comparable levels of intensity in all lanes. Moreover, re-analysis of nuclear extracts by EMSA displayed equal levels of Oct-1-DNA binding complexes that served as a loading control (data not shown).

To determine whether the PTEN effects were specific only to LNCaP cells, we analyzed two other human prostate cell lines, PC-3 and DU-145. PC-3 cells contain not only constitutive Akt activity due to the loss of PTEN function (40, 41) but also display constitutive IKK activation, DNA binding, and NF-κB-dependent transcription (52). On the other hand, DU-145 cells express a functional PTEN protein (41) and consequently do not display constitutive Akt activity. In this way, we could determine whether PTEN expression blocked either basal or TNF-induced nuclear translocation in either PC-3 or DU-145 cells. PC-3 and DU-145 were infected with Ad-GFP, Ad-PTEN, or Ad-SRIκBα, and nuclear extracts were harvested following TNF stimulation. Interestingly, expression of PTEN did not block the high basal NF-κB DNA binding activity normally observed in PC-3 cells nor did it inhibit TNF-induced increases in NF-κB binding, as compared with Ad-GFP-infected control cells (Fig. 3, *left panel*). Ectopic expression of PTEN also did not inhibit TNF-induced NF-κB nuclear DNA binding activity in DU-145 cells (Fig. 3, *right panel*). However, expression of the SR-IκBα protein blocked basal NF-κB DNA binding activity in PC-3 cells and also inhibited TNF-induced NF-κB activity in both PC-3 and DU-145 cells (Fig. 3). The inability of PTEN to block TNF-induced nuclear translocation signals was not due to differences in adenoviral mediated expression of PTEN, because PTEN was effectively expressed in both PC-3 and DU-145 cells, and constitutively active phospho-Akt was down-regulated in PC-3 cells (data not shown). We found that TNF weakly stimulated Akt activity in DU-145 cells, and the expression of PTEN or exposure to the PI3K inhibitor, LY294002, blocked TNF-induced NF-κB transcriptional activity in transient reporter assays. However, consistent with the data here, LY294002 failed to block TNF-induced nuclear translocation of

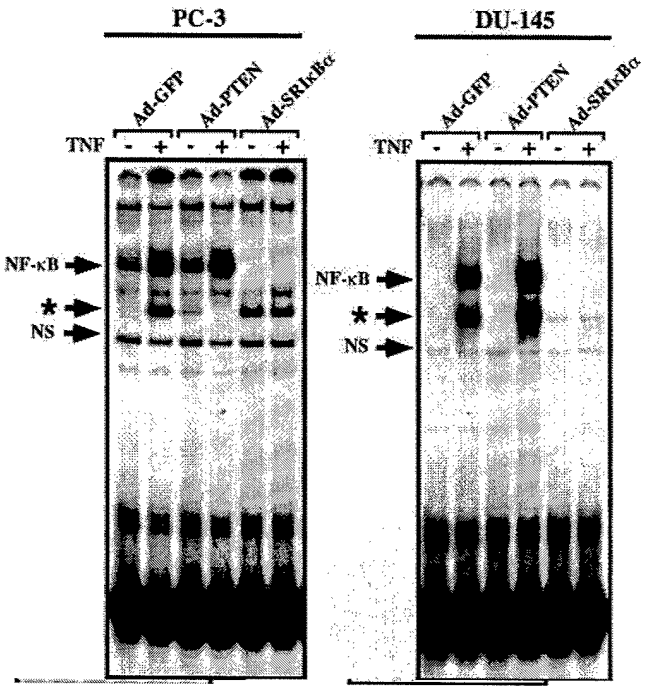


FIG. 3. **Expression of PTEN fails to block TNF-induced nuclear translocation and DNA binding activity of NF-κB in PC-3 and DU-145 prostate cells.** PC-3 and DU-145 cells were infected with Ad-GFP, Ad-PTEN, or Ad-SRIκBα virus (50 pfu/cell). Twenty four hours following infection, cells were either left untreated or stimulated for 30 min with TNF (10 ng/ml), and nuclear extracts were isolated and analyzed by EMSA. *Arrows* identify the NF-κB-DNA binding complex (composed of p65 and p50), the p50 homodimer (indicted by an *asterisk*), and a nonspecific (*NS*) band that demonstrates that equal amounts of total extract were analyzed in each lane.

NF-κB in DU-145 cells, as measured by EMSA (data not shown). PTEN did not affect TNF-induced nuclear translocation and DNA binding of NF-κB (composed of p65 and p50 heterodimer) in LNCaP, PC-3, or DU-145 cells. Therefore, despite PC-3 cells displaying a loss of p50 homodimer binding (compare Fig. 2*F* with Fig. 3), we predict that this effect could not account for the ability of PTEN to inhibit NF-κB-dependent transcriptional activity. Collectively, these results indicate that the expression of PTEN in LNCaP, PC-3, or DU-145 cells failed to block NF-κB DNA binding, suggesting that PTEN functions to block NF-κB-dependent gene expression through an alternative mechanism.

PTEN Regulates NFκB by Repressing the Transactivation Domain of the p65 Subunit—To determine whether PTEN could modulate NF-κB by blocking the transactivation function of p65, we utilized a plasmid encoding a Gal4-p65 fusion protein. In this fusion protein, sequences encoding the DNA binding domain of the yeast Gal4 transcription factor have been joined with sequences encoding the transactivation domain I of p65 (43). Experiments were performed by co-transfecting cells with an expression plasmid encoding Gal4-p65 and with a Gal4-responsive luciferase reporter (Gal4-Luc). In addition to analyzing p65, we also evaluated whether PTEN could modulate the transactivation domain of CREB, a transcription factor known to be directly phosphorylated by Akt (53), and c-Jun. As shown in Fig. 4*A*, the transactivation potential of p65, CREB, and c-Jun was increased in LNCaP cells following the addition of TNF. The expression of PTEN blocked the ability of TNF to stimulate the transactivation domain of p65 and CREB (Fig. 4*A*). Interestingly, PTEN expression did not inhibit the ability of TNF to stimulate the transactivation domain of c-Jun (Fig. 4*A*). These results suggest that PTEN inhibits TNF-induced NF-κB activation by blocking the transactivation potential of

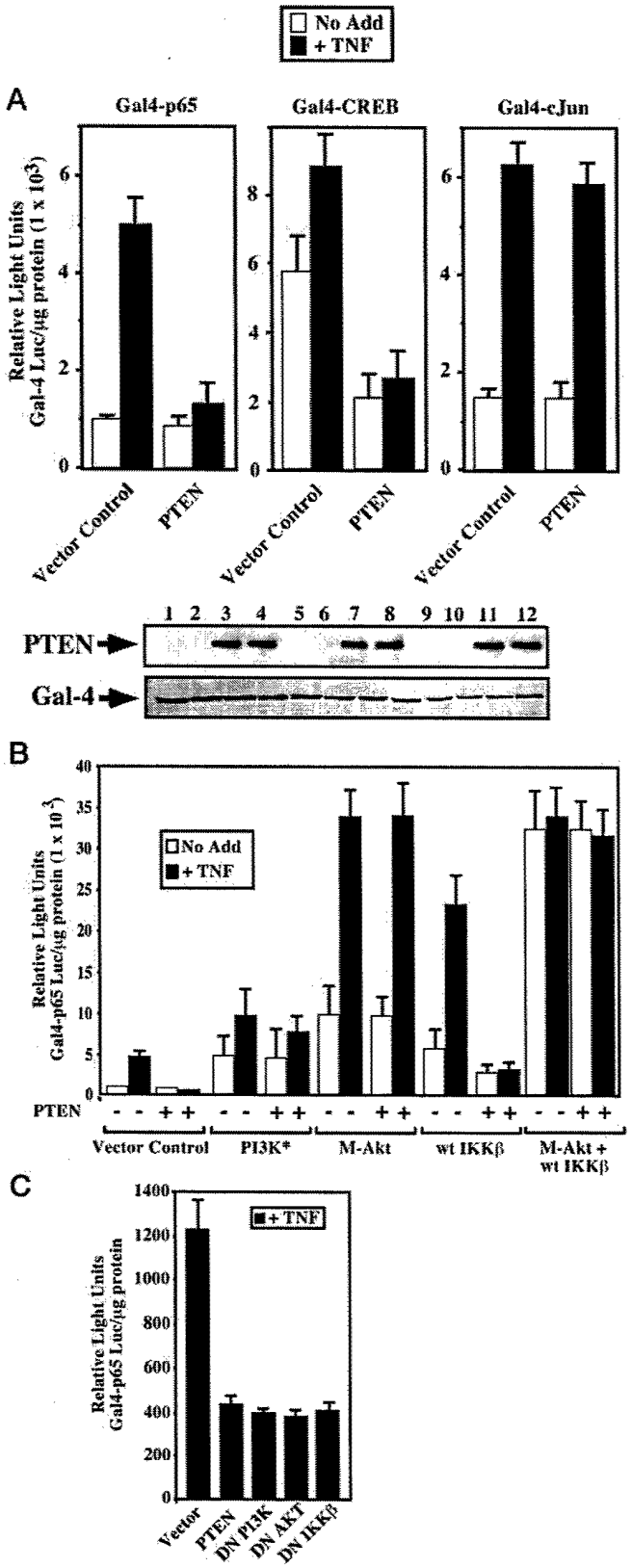


FIG. 4. **PTEN blocks TNF-induced activation of NF-κB by modulating the transactivation domain of p65.** *A*, PTEN inhibits the ability of TNF to stimulate the transactivation domain of the p65 subunit of NF-κB. LNCaP cells were co-transfected with plasmids encoding the Gal4-p65, Gal4-CREB, or Gal4-cJun fusion proteins and with the 4×Gal4-Luc reporter. In addition, cells were transfected with either the empty vector control or with an expression vector encoding wild-type PTEN. Eighteen hours following transfection, cells were either left untreated or were stimulated with TNF (10 ng/ml). Eighteen hours following stimulation, cells were harvested, and luciferase activities were analyzed. Data presented represent the mean ± S.D. of three independent experiments. *Bottom panel*, Western blot analysis of cell extracts demonstrate PTEN and Gal-4 protein expression. *B*, activated forms of PI3K and Akt proteins overcome the ability of PTEN to block TNF-induced stimulation of the p65 transactivation domain. LNCaP cells were co-transfected with Gal4-p65, Gal4-Luc reporter, and with plasmids encoding activated PI3K, Akt, IKKβ, Akt, and IKKβ or empty vector control. Additionally, cells were co-transfected with plasmid encoding PTEN or empty vector control. Eighteen hours following transfection cells were either left untreated or were stimulated with TNF (10 ng/ml). Cell extracts were harvested 18 h following the addition of TNF, and equal amounts of protein lysates were assayed for luciferase activity. Data represent the mean ± S.D. of two individual experiments performed in triplicate. *C*, the ability of TNF to stimulate the p65 transactivation domain is blocked by PTEN and dominant negative forms of PI3K, Akt, and IKK. LNCaP cells were co-transfected with Gal4-p65, Gal4-Luc reporter, and with expression plasmids encoding PTEN, dominant negative (*DN*) PI3K (Δp85), Akt(Lys → Met), IKKβ (SS ≫ AA). Eighteen hours following transfection cells were stimulated with TNF, and 18 h following the addition of TNF, cells were harvested, and luciferase activities were determined. Results represent three separate experiments performed in triplicate, and the mean ± S.D. are shown.

p65 and that down-regulation of PIP_3 by PTEN expression also modulates CREB transactivation function.

To understand better the signaling pathways by which PTEN inhibits the transactivation potential of p65, we investigated whether active PI3K or Akt proteins could rescue PTEN-dependent inhibition in response to TNF. As shown in Fig. 4*B*, the expression of either PI3K or Akt proteins alone was capable of up-regulating the transactivation potential of p65 above vector control plasmid levels in unstimulated LNCaP cells. This effect was further elevated following the addition of TNF (Fig. 4*B*). PTEN expression failed to inhibit the ability of constitutively active PI3K to target the transactivation domain of p65 following TNF stimulation (Fig. 4*B*). Moreover, PTEN expression was unable to block the synergistic activation induced by M-Akt following TNF stimulation (Fig. 4*B*). Therefore, these results suggest that M-Akt can bypass the requirement for PIP_3 activity, although TNF still provides an inducible signal (Fig. 4*B*).

Several reports including our own (15, 30, 36) have indicated that IKK participates in targeting the p65 transactivation domain in response to activated Akt. Recently, we have shown (36) that serine residues 529 and 536, located in the transactivation domain of p65 and shown to be targeted by CK II and IKK, respectively, are both required for Akt-induced activation of NF-κB. Therefore, although in Fig. 2*D* we demonstrate that PTEN did not block the ability of IKK to phosphorylate IκBα following TNF stimulation, additional transfection experiments were performed to determine whether IKK alone was enough to target the transactivation domain of p65 following TNF stimulation. As shown in Fig. 4*B*, the expression of IKKβ activated the transactivation domain of p65 in unstimulated LNCaP cells, which was further enhanced following TNF stimulation. PTEN was capable of inhibiting TNF-stimulated LNCaP cells expressing wild-type IKKβ (Fig. 4*B*). These results suggest that in order for TNF to up-regulate the transactivation domain of p65 in LNCaP cells, both Akt and IKK are required. This hypothesis is supported by the observation that co-expression of both M-Akt and IKKβ strongly activates the p65 transactivation domain, without the need for TNF stimulation (Fig. 4*B*). Moreover, this activation could not be inhibited by the expression of PTEN (Fig. 4*B*).

To determine whether Akt and IKK are required for TNF-dependent signals to target the transactivation potential of the p65 subunit of NF-κB, LNCaP cells were co-transfected with Gal4-p65, the Gal4 reporter, and plasmids encoding for various dominant negative proteins. As shown in Fig. 4*C*, cells express-

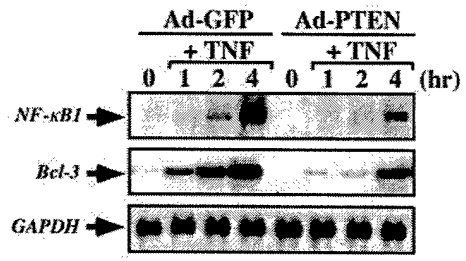


FIG. 5. **Reintroduction of PTEN into LNCaP cells blocks TNF-induced NF-κB-dependent gene expression.** LNCaP cells were infected with either Ad-GFP or Ad-PTEN. Twenty four hours following infection cells were either left untreated or were stimulated with TNF. Total RNAs were isolated, resolved on a formaldehyde gel, and blotted onto nitrocellulose. Gene expression for *NF-κB1*, *bcl*-3, and *GAPDH* were determined by analyzing Northern blots with radiolabeled cDNAs corresponding to these genes. Equal amounts of RNA were loaded in each lane because no differences were observed for GAPDH expression.

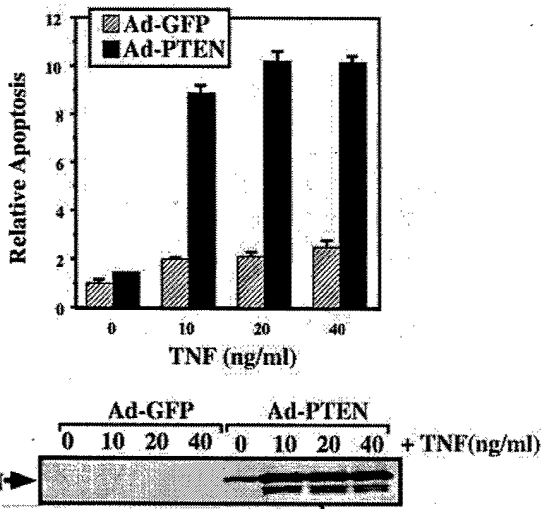


FIG. 6. **Ectopic expression of PTEN sensitizes LNCaP cells to TNF-induced apoptosis.** Subconfluent LNCaP cells were infected with either Ad-GFP or Ad-PTEN (10 pfu/cell) 24 h prior to the addition of varying concentrations of TNF (0, 10, 20, and 40 ng/ml). Twenty four hours following the addition of TNF, adherent and non-adherent cells were harvested and assayed for the presence of histone-associated DNA fragments using the Cell Death Detection ELISA kit (Roche Molecular Biochemicals). Relative apoptosis was plotted, where Ad-GFP-infected LNCaP cells grown in the absence of TNF was normalized to one. Data presented were performed in duplicate, and similar results were obtained in three independent experiments. Western blot analysis for PTEN expression demonstrated transgene expression over the 48-h time course of the experiment.

ing plasmids encoding PTEN or dominant negative PI3K, Akt, or IKKβ all blocked the ability of TNF to stimulate the transactivation domain of p65 in LNCaP cells. Similar results were observed in PC-3 cells, where expression of PTEN or dominant negative constructs encoding PI3K, Akt, or IKKβ inhibited the basal transactivation potential of the p65 subunit. The ability of PTEN to inhibit the p65 transactivation domain was not specific only to TNF, as this effect was also observed for interleukin-1β (data not shown). These results indicate that PTEN modulates NF-κB transcriptional activity through a mechanism that controls the transactivation function of the p65 subunit and not by inhibiting signals that control nuclear translocation or DNA binding of NF-κB.

The Ability of PTEN to Block the Transactivation Potential of the p65 Subunit of NFκB Is Associated with a Loss of NF-κB-dependent Gene Expression—Our results suggest that the ability of PTEN to inhibit PIP_3 and down-regulate Akt activity alone is enough to inhibit the transactivation potential of the p65 subunit, which would be predicted to block endogenous NF-κB-dependent gene expression (Fig. 4). To determine whether the expression of PTEN blocked NF-κB-dependent gene expression following TNF stimulation, we analyzed the expression of two NF-κB regulated genes, *NF-κB1* and *bcl*-3 (54, 55). LNCaP cells were infected with either Ad-PTEN or Ad-GFP control virus, RNAs were isolated, and Northern blot analysis was performed. As shown in Fig. 5, LNCaP cells infected with control virus displayed an increase in both *NF-κB1* and *bcl*-3 transcripts by 2 h, which persisted for 4 h following TNF stimulation. Consistent with the ability of PTEN to block NF-κB transcriptional activity in transient reporter assays, LNCaP cells expressing PTEN displayed a significant decrease in both *NF-κB1* and *bcl*-3 transcripts (Fig. 5). The differences in gene expression were not due to uneven loading of RNAs, because re-analysis of blots for *GAPDH* displayed equal levels of transcripts (Fig. 5). These results are consistent with our finding that the ability of PTEN to down-regulate the transactivation domain of p65 following TNF stimulation is associated with a loss of NF-κB-dependent gene expression.

PTEN Potentiates TNF-induced Apoptosis—Thus far, we have established that the re-introduction of the PTEN tumor suppressor protein into LNCaP cells affects the ability of these cells to respond to TNF-induced NF-κB transcriptional activation. Since it has been well established that TNF-induced activation of NF-κB is required to overcome the ability of this cytokine to induce apoptosis (56–61), we next investigated the effect of PTEN on cellular sensitivity to TNF-induced apoptosis. As shown in Fig. 6, LNCaP cells became markedly sensitized to TNF-induced apoptosis following Ad-PTEN infection at a dose as low as 10 ng/ml TNF for 24 h, as compared with LNCaP cells infected with the Ad-GFP control. The histone-associated DNA fragments detected using the Cell Death Detection ELISA (Fig. 6), were also confirmed using standard agarose gel electrophoresis analysis (data not shown). Collectively, these results indicate that ectopic expression of PTEN in LNCaP cells sensitized these cells to TNF-induced apoptosis. This work is consistent with the idea that modulation of NF-κB transcriptional activity by the ability of PTEN to down-regulate Akt activity sensitizes the LNCaP cell line to TNF-induced apoptosis.

DISCUSSION

In this report, we demonstrate that the down-regulation of Akt activity by the re-introduction of the PTEN tumor suppressor protein in prostate epithelial cells inhibits the ability of TNF to stimulate NF-κB-dependent transcription. The ability of PTEN to block TNF-induced activation of NF-κB is dependent on the lipid phosphatase activity of PTEN and on the concomitant down-regulation of Akt activity. In support of this, we found that constitutively active Akt overcame the ability of PTEN to suppress NF-κB activation following TNF stimulation. CaMKK, which has been shown to activate Akt through PI3K-independent pathways (48), was capable of activating NF-κB even in the presence of PTEN. In our model system, the inhibition of Akt activity by the expression of PTEN did not block TNF-induced IKK activation, IκBα degradation, p105 processing, p65 nuclear translocation, or DNA binding of NF-κB. Rather, cells expressing PTEN displayed equal or even better NF-κB DNA binding activities following TNF stimulation, as compared with cells expressing GFP control protein. To determine mechanistically how PTEN-dependent inhibition of Akt blocked the ability of TNF to stimulate NF-κB, we evaluated whether PTEN down-regulated the transactivation potential of the p65 subunit of NF-κB. We found that PTEN inhibited the ability of TNF to stimulate the transactivation potential of p65, as well as CREB, but not c-Jun. The transactivation potential of p65 following TNF stimulation could be rescued from

PTEN-dependent repression by re-introducing activated forms of PI3K and Akt or Akt and IKK. The ability of TNF to stimulate the transactivation domain of p65 was not only blocked by PTEN but also by the expression of dominant negative forms of PI3K, Akt, or IKKβ proteins. These results suggest that all of these signaling molecules are important for full NF-κB-dependent transcriptional activity. Modulation of the transactivation function of p65 by PTEN is important for NF-κB-dependent transcription, because PTEN blocked TNF-induced up-regulation of *NF-κB1* and *bcl-3* transcripts. Consistent with a previous report (16) that used a PI3K inhibitor, we find that expression of PTEN results in a loss of Akt and NF-κB activities and sensitizes LNCaP cells to TNF-induced apoptosis.

During the preparation of this manuscript, two other independent reports were published that address the effect of the *PTEN* tumor suppressor gene expression on NF-κB activation following stimulation by proinflammatory cytokines (38, 39). The basic conclusion in both of these studies was that PTEN was capable of inhibiting NF-κB-dependent gene expression in transient reporter gene assays. However, some major discrepancies exist between our results and the other reported molecular mechanisms by which PTEN inhibited NF-κB (38, 39). In the other studies, it was argued that expression of PTEN, either transiently or stably, results in a loss of NF-κB DNA binding potential. Under no circumstances did we observe this effect in any of the cell lines we tested. Rather, as shown in Figs. 2*E* and 3, expression of PTEN results in a slight increase in NF-κB DNA binding following TNF stimulation. This effect was not specific to TNF, because PTEN also failed to block NF-κB-DNA binding activity in LNCaP cells following IL-1β stimulation.[2] Koul *et al.* (38) also indicated that PTEN expression down-regulated the p50:p50 homodimer DNA-binding complex of NF-κB. Although we did observe this effect in PC-3 cells, this mechanism did not account for PTEN-dependent inhibition of NF-κB activity and was not observed in either LNCaP or DU-145 cells (Fig. 2*E* and 3). In contrast, in our model system PTEN did not block TNF-induced DNA binding of the transcriptionally active p65:p50 heterodimer of classical NF-κB. Gustin *et al.* (39) indicated that PTEN expression blocks the ability of the IKK complex to stimulate IκBα phosphorylation. Our data strongly indicate that this is not the case in LNCaP, PC-3, or DU-145 cells. On the contrary, we observed instead a normal increase in IκBα phosphorylation and degradation and nuclear translocation of p65 (Fig. 2, *B*, *D*, and *E* and data not shown). Moreover, LNCaP cells expressing PTEN display normal TNF-induced IKK activity, indicating that PTEN does not block the ability of IKK to phosphorylate IκBα (Fig. 2*C*). If PTEN was functioning to inhibit the ability of the IKK complex to phosphorylate IκB, then one would predict that PTEN-deficient cells would maintain constitutive Akt phosphorylation and IKK activity and display constitutive NF-κB DNA binding. However, these results are not what was observed in LNCaP cells nor what was reported for PTEN null MEF cells (6). In contrast, cells constitutively expressing active Akt still require a stimulus to induce IκB degradation, DNA binding, and NF-κB-dependent gene expression. This is supported not only in our study, but also in other recent reports (38, 39). Our results and the results of others (38) indicate that constitutively active endogenous Akt alone is not enough to activate effectively the IKK-dependent pathways. Conversely, the inhibition of Akt via PTEN is incapable of fully inhibiting IKK activity and IκBα phosphorylation and degradation (Fig. 2, *B* and *C*) (38).

The ability of PTEN to block NF-κB-dependent gene expression and sensitize cells to TNF-induced apoptosis may allow insight into the use of pharmacological inhibitors of the PI3K pathway to therapeutically treat human tumors that have lost functional PTEN expression. NF-κB is known to protect cells from apoptosis by up-regulating target genes that restrict cytochrome *c* release from the mitochondria and inhibit caspase activation. To date, these NF-κB-regulated genes include *cIAP-1*, *cIAP-2*, *XIAP*, *Bcl-X_L*, *A1/Bfl-1*, *Nrl3*, *IEX-1L*, and the recently discovered *NDED* (61, 62). Although the down-regulation of PI3K activity has been shown to induce apoptosis that can be rescued by the overexpression of the p65 subunit of NF-κB (16), we cannot exclude the possibility that PTEN induces cell death through other mechanisms as well. For example, as we have described here, another pro-survival transcription factor, CREB, is also negatively regulated by PTEN activity. Huang *et al.* (63) recently reported similar results indicating that PTEN-mediated repression of phosphorylation of CREB at serine 133 was associated with the transcriptional down-regulation of the *bcl-2* protooncogene in human prostate cells. Because Akt has been shown recently (64) to provide protection from the TRAIL/Apo-2L pathway by inhibiting BID cleavage, it is possible that the inhibition of Akt via PTEN expression would sensitize prostate cells to similar death pathways initiated by TNF. Moreover, because Bcl-2 and Bcl-X_L block TRAIL-induced apoptosis, it could be that a combination of these different signaling events is required to allow PTEN to sensitize prostate cells to apoptotic stimuli.

Acknowledgments—We thank Dr. Lily Wu (UCLA) for Ad-GFP and for help with the construction of Ad-PTEN virus. We thank Dr. Tony Means (Duke University, Durham, NC) for kindly providing activated CAMKK. We also thank Laurey Comeau for the excellent technical support with this project.

REFERENCES

1. Vazquez, F., and Sellers, W. R. (2000) *Biochim. Biophys. Acta* **1470,** M21–M35
2. Di Cristofano, A., and Pandolfi, P. P. (2000) *Cell* **100,** 387–390
3. Leevers, S. J., Vanhaesebroeck, B., and Waterfield, M. D. (1999) *Curr. Opin. Cell Biol.* **11,** 219–225
4. Maehama, T., and Dixon, J. E. (1999) *Trends Cell Biol.* **9,** 125–128
5. Cantley, L. C., and Neel, B. G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4240–4245
6. Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P., and Mak, T. W. (1998) *Cell* **95,** 29–39
7. Myers, M. P., Pass, I., Batty, I. H., Van der Kaay, J., Stolarov, J. P., Hemmings, B. A., Wigler, M. H., Downes, C. P., and Tonks, N. K. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13513–13518
8. Maehama, T., and Dixon, J. E. (1998) *J. Biol. Chem.* **273,** 13375–13378
9. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) *Cell* **91,** 231–241
10. Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. (1998) *Science* **282,** 1318–1321
11. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) *Cell* **96,** 857–868
12. Sabbatini, P., and McCormick, F. (1999) *J. Biol. Chem.* **274,** 24263–24269
13. Gottlob, K., Majewski, N., Kennedy, S., Kandel, E., Robey, R. B., and Hay, N. (2001) *Genes Dev.* **15,** 1406–1418
14. Romashkova, J. A., and Makarov, S. S. (1999) *Nature* **401,** 86–89
15. Madrid, L., Wang, C.-Y., Guttridge, D. C., Schottelius, A. J. G., Baldwin, A. S., Jr., and Mayo, M. W. (2000) *Mol. Cell. Biol.* **20,** 1626–1638
16. Reddy, S. A. G., Huang, J. H., and Liao, W. S.-L. (2000) *J. Immunol.* **164,** 1355–1363
17. Yang, C. H., Murti, A., Pfeffer, S. R., Kim, J. G., Donner, D. B., and Pfeffer, L. M. (2001) *J. Biol. Chem.* **276,** 13756–13761
18. Baldwin, A. (1996) *Annu. Rev. Immunol.* **14,** 649–681
19. Mayo, M. W., and Baldwin, A. S. (2000) *Biochim. Biophys. Acta* **1470,** M55–M62
20. Zandi, E., and Karin, M. (1999) *Mol. Cell. Biol.* **19,** 4547–4551
21. Ghosh, S., May, M., and Kopp, E. B. (1998) *Annu. Rev. Immunol.* **16,** 225–260
22. May, M. J., and Ghosh, S. (1998) *Immunol. Today* **19,** 80–88
23. Zhong, H., SuYang, H., Erdjument-Bromage, H., Tempst, P., and Ghosh, S. (1997) *Cell* **89,** 413–424
24. Zhong, H., Voll, R. E., and Ghosh, S. (1998) *Mol. Cell* **1,** 661–671
25. Wang, D., Westerheide, S. D., Hanson, J. L., and Baldwin, A. S., Jr. (2000) *J. Biol. Chem.* **275,** 32592–32597
26. Mercurio, F., Zhu, H., Murray, B. W., Shevchenko, A., Bennett, B. L., Li, J., Young, D. B., Barbosa, M., Mann, M., Manning, A., and Rao, A. (1997) *Science* **278,** 860–866
27. Sakurai, H., Chiba, H., Miyoshi, H., Sugita, T., and Toriumi, W. (1999) *J. Biol. Chem.* **274,** 30353–30356

[2] M. W. Mayo, unpublished observations.

28. Hoeflich, K. P., Luo, J., Rubie, E. A., Tsao, M. S., Jin, O., and Woodgett, J. R. (2000) *Nature* **406,** 86–90
29. Bonnard, M., Mirtsos, C., Suzuki, S., Graham, K., Huang, J., Ng, M., Itie, A., Wakeham, A., Shahinian, A., Henzel, W. J., Elia, A. J., Shillinglaw, W., Mak, T. W., Cao, Z., and Yeh, W. C. (2000) *EMBO J.* **19,** 4976–4985
30. Sizemore, N., Leung, S., and Stark, G. R. (1999) *Mol. Cell. Biol.* **19,** 4798–4805
31. Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. (1999) *Nature* **401,** 82–85
32. Kane, L. P., Shapiro, V. S., Stokoe, D., and Weiss, A. (1999) *Curr. Biol.* **9,** 601–604
33. Pan, Z. K., Christiansen, S. C., Ptasznik, A., and Zuraw, B. L. (1999) *J. Biol. Chem.* **274,** 9918–9922
34. Beraud, C., Henzel, W. J., and Baeuerle, P. A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 429–434
35. Sontag, E., Sontag, J.-M., and Garcia, A. (1997) *EMBO J.* **16,** 5662–5671
36. Madrid, L. V., Mayo, M. W., Reuther, J. Y., and Baldwin, A. S., Jr. (2001) *J. Biol. Chem.* **276,** 18934–18940
37. Delhase, M., Li, N., and Karin, M. (2000) *Nature* **406,** 367–368
38. Koul, D., Yao, Y., Abbruzzese, J. L., Yung, A. W. K., and Reddy, S. A. G. (2001) *J. Biol. Chem.* **276,** 11402–11408
39. Gustin, J. A., Maehama, T., Dixon, J. E., and Donner, D. B. (2001) *J. Biol. Chem.* **276,** 27740–27744
40. Vlietstra, R. J., van Alewijk, D. C., Hermans, K. G., van Steenbrugge, G. J., and Trapman, J. (1998) *Cancer Res.* **58,** 2720–2723
41. Wu, X., Senechal, K., Neshat, M. S., Whang, Y. E., and Sawyers, C. L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 15587–15591
42. Mayo, M. W., Norris, J. L., and Baldwin, A. S. (2001) *Methods Enzymol.* **333,** 73–87
43. Schmitz, M. L., and Baeuerle, P. A. (1991) *EMBO J.* **10,** 3805–3817
44. Chatila, T., Anderson, K. A., Ho, N., and Means, A. R. (1998) *J. Biol. Chem.* **271,** 21542–21548
45. Mayo, M. W., Wang, C.-Y., Cogswell, P. C., Rogers-Graham, K. S., Lowe, S. W., Der, C. J., and Baldwin, A. S. (1997) *Science* **278,** 1812–1815
46. Gomez-Foix, A. M., Coats, W. S., Baque, S., Alam, T., Gerard, R. D., and Newgard, C. B. (1992) *J. Biol. Chem.* **267,** 25129–25134
47. Furnari, F. B., Huang, H. J., and Cavenee, W. K. (1998) *Cancer Res.* **58,** 5002–5008
48. Yano, S., Tokumitsu, H., Soderling, T. R. (1998) *Nature* **396,** 584–587
49. Heissmeyer, V., Krappmann, D., Wulczyn, F. G., and Scheidereit, C. (1999) *EMBO J.* **18,** 4766–4778
50. Heissmeyer, V., Krappmann, D., Hatada, E. N., and Scheidereit, C. (2001) *Mol. Cell. Biol.* **21,** 1024–1035
51. Salmeron, A., Janzen, J., Soneji, Y., Bump, N., Kamens, J., Allen, H., and Ley, S. C. (2001) *J. Biol. Chem.* **276,** 22215–22222
52. Palayoor, S. T., Youmell, M. Y., Calderwood, S. K., Coleman, C. N., and Price, B. D. (1999) *Oncogene* **18,** 7389–7394
53. Du, K., and Montminy, M. (1998) *J. Biol. Chem.* **273,** 32377–32379
54. Cogswell, P. C., Scheinman, R. I., and Baldwin, A. S. (1993) *J. Immunol.* **7,** 2794–2804
55. Brasier A. R., Lu, M., Hai, T., Lu, Y., and Boldogh, I. (2001) *J. Biol. Chem.* **276,** 32080–32093
56. Liu, Z.-G., Hsu, H., Goeddel, D. V., and Karin, M. (1996) *Cell* **87,** 565–576
57. Wang, C.-Y., Mayo, M. W., and Baldwin, A. S. (1996) *Science* **274,** 784–787
58. Beg, A. A., and Baltimore, D. (1996) *Science* **274,** 782–784
59. Van Antwerp, D. J., Martin, S., Kafri, T., Green, D. R., and Verma, I. M. (1996) *Science* **274,** 787–789
60. Wang, C.-Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S. (1998) *Science* **281,** 1680–1683
61. You, Z., Ouyang, H., Lopatin, D., Polver, P. J., and Wang, C.-Y. (2001) *J. Biol. Chem.* **276,** 26398–26404
62. Pahl, H. L. (1999) *Oncogene* **18,** 6853–6866
63. Huang, H., Cheville, J. C., Pan, Y., Roche, P. C., Schmidt, L. J., and Tindall, D. J. (2001) *J. Biol. Chem.* **276,** 38830–38836
64. Thakkar, H., Chen, X., Tyan, F., Gim, S., Robinson, H., Lee, C., Pandey, S. K., Nwokorie, C., Onwudiwe, N., and Srivastava, R. K. (2001) *J. Biol. Chem.* **276,** 38361–38369

The PTEN tumor suppressor is a negative modulator of androgen receptor transcriptional activity.

Bicheng Nan[1,2], Thiti Snabboon[1,2], Emmanual Unni[1,2], Xiu-Juan Yuan[3], Young E. Whang[3,6], Marco Marcelli[1,2,4,5,6]

Department of Medicine[1] and Molecular and Cellular Biology[4], Baylor College of Medicine and VA Medical Center[2], Houston TX 77030, Lineberger Comprehensive Cancer Center[3], University of North Carolina School of Medicine, Chapel Hill, NC 27599.

[5]To whom correspondence should be addressed at: 2002 Holcombe Blvd., Houston TX 77030; Phone 713 794 7945, Fax 713 794 7714; e-mail: marcelli@bcm.tmc.edu

[6]These two authors share senior authorship of the paper

Running title: AR-PTEN interaction in prostate cancer

Key words: AR, PTEN, prostate cancer.

Supported by grants from the Department of Defense Prostate Cancer Research Program (DAMD17-00-1-0037) and NIH (CA85772) to Y.E.W., and Department of Defense Prostate Cancer Research (DAMD17-00-1-0075) and Merit Review Program of the Veterans Administration to M.M.

Abstract

To investigate whether the tumor suppressor gene PTEN affects the activity of the androgen receptor (AR), we monitored the expression of the apoptotic gene Bax (inserted in an adenovirus where it is driven by the androgen receptor responsive promoter ARR_2PB) in the presence or absence of dihydrotestosterone, in PTEN (+) or (-) prostate cancer cell lines, infected with an adenovirus containing wild type PTEN (Av-CMV-PTEN) or a control LacZ-expressing construct. Our results showed that AR transcriptional activity was antagonized by PTEN expression. This antagonism was not cell line dependent, as it was observed in both LNCaP and LAPC-4 cells, or promoter dependent, as it was observed for a reporter gene (Bax) driven by an exogenous androgen-responsive promoter (the ARR_2PB promoter), and for a native gene (PSA) driven by an endogenous AR-responsive promoter. Additional experiments performed with viruses containing constitutively active (Adeno-myrAkt) or dominant negative (Adeno-dnAkt) forms of Akt demonstrated that Akt, a protein kinase whose activation is known to be inhibited by PTEN, mediated the observed antagonism between PTEN and AR transcriptional activity. Recently two putative Akt phosphorylation sites have been identified in the AR sequence. Site directed mutagenesis was utilized to convert these two serine into alanine residues. The resulting construct, named CMV-AR S213A&S791A was transfected in AR (-) and PTEN **(-)** PC-3 cells in the presence or absence of Av-CMV-PTEN and of a reporter plasmid (GRE_2E1b-Luc) containing the luciferase gene driven by a well-characterized androgen responsive promoter. These experiments demonstrated that similarly to the wt molecule, AR S213A&S791A was transcriptionally inhibited by PTEN, suggesting that Akt does not have an effect on AR

transcription by direct phosphorylation, but probably by affecting the availability of a downstream molecule whose main mechanism of action is that of modulating AR transcription. The data presented in this communication suggest that loss of PTEN function may facilitate activation of AR signaling and progression to androgen independence in prostate cancer.

INTRODUCTION

The widespread use of prostate specific antigen (PSA) has significantly increased our ability to correctly identify patients affected by prostate cancer (CaP). This powerful diagnostic tool has changed the epidemiology of CaP and an increasing number of patients are now diagnosed with organ-confined disease (Hankey *et al.*, 1999). In addition, overall death rates are falling in many industrialized countries due to early diagnosis (Oliver *et al.*, 2001). Despite these encouraging statistics, prostate cancer is still the most frequently diagnosed visceral cancer in American men, and there will be an estimated 189,000 new cases and 30,200 deaths from it in 2002 (Jemal *et al.*, 2002; Schroder, 1999).

CaP can be eradicated when organ-confined, but systemic disease is incurable. Systemic prostate cancer is usually treated with hormonal ablative therapy, but virtually all patients receiving this treatment relapse and develop androgen-independent tumors for which only experimental treatments exist (Schroder, 1999). Urgently needed is a better understanding of prostate cancer progression to androgen-independence at the molecular level, in order to identify new targets for novel therapy design.

Centrally located in the pathway activated by circulating androgens is the androgen receptor (AR), a member of the nuclear receptor family. After binding ligand, this molecule becomes activated with an associated change in conformation, translocates to the nucleus and binds DNA, ultimately regulating the transcription of androgen-responsive target genes (Balk, 2002). In the prostate, AR is believed to work by stimulating activities which antagonize apoptosis and induce cell proliferation (Denmeade *et al.*, 1996). AR is expressed in a normal or amplified way in patients with

androgen-independent disease, and mutations of its ligand-binding domain have been described which expand binding specificities (Taplin *et al.*, 1999; Van-der-Kwast *et al.*, 1991; Visakorpi *et al.*, 1995) and are associated with disease progression. Nevertheless, the large majority of AR analyzed at the molecular level does not contain mutations (Marcelli *et al.*, 2000), and so other mechanisms must be involved with progression to androgen-independent disease.

According to a recent paper, presence of AR is essential for androgen independent CaP cells proliferation (Zegarra-Moro *et al.*, 2002). In addition, AR expression level increases in androgen independent prostate cancer (Balk, 2002). Therefore, to reconcile the apparent contradiction that AR is essential for proliferation of androgen-independent prostate cancer cells (Zegarra-Moro *et al.*, 2002) but use of AR antagonists in association with inhibitors of testosterone synthesis is ineffective in patients with androgen independent disease (Eisenberger *et al.*, 1998), many authors have hypothesized that AR can function in a ligand-independent way. In support of this hypothesis, a wide body of literature has been published demonstrating that AR can be activated by mechanisms involving protein kinase A, or tyrosine kinase receptors pathways (Craft *et al.*, 1999; Culig *et al.*, 1994; Nazareth and Weigel, 1996; Ueda *et al.*, 2002a; Ueda *et al.*, 2002b; Yeh *et al.*, 1999).

A frequent molecular abnormality detected in advanced prostate cancer consists in loss of the tumor suppressor gene PTEN (Dong *et al.*, 2001; McMenamin *et al.*, 1999; Suzuki *et al.*, 1998). This molecule works by antagonizing the PI3K pathway to induce apoptosis and growth arrest (Cantley, 2002). The predominant enzymatic activity of PTEN consists in dephosphorylating the glycerophospholipid phosphatidylinositol 3,4,5-

triphosphate (PI3,4,5,P3) at the D3 position to form phosphatidylinositol 4,5-biphosphate (PI4,5,P2) (Vivanco and Sawyers, 2002). PI3,4,5P3, the main substrate formed after activation of the phosphatidylinositol 3-kinase (PI3K) pathway, is essential to achieve activation of the serine/threonine kinase Akt by anchoring it to the inner surface of the cell membrane through its PH (pleckstrin homology) domain (Andjelkovic *et al.*, 1997). Once anchored to the plasma membrane, Akt achieves its final active state through phosphorylation at threonine 308 by 3-phosphoinositide dependent protein kinase 1 (PDK1) (Vanhaesebroeck and Alessi, 2000), and at Ser473 by PDK2 (Vanhaesebroeck and Alessi, 2000). Thus, the main mechanism through which PTEN exerts tumor suppression consists in antagonizing PI3,4,5P3 formation, and thus preventing Akt activation (Stocker *et al.*, 2002), which signals survival and mitogenesis to the cell.

PTEN and AR play opposing roles in the prostate (AR induces proliferation and antiapoptosis (Denmeade *et al.*, 1996), while PTEN, induces apoptosis and growth arrest (Yuan and Whang, 2002)). Previous studies have linked PTEN (and Akt signaling) and AR activity, but the conclusions are controversial, as Li and collaborator have shown that PTEN (through down regulation of Akt) works as an antagonist of AR activity (Li *et al.*, 2001a), while Lin and collaborator have provided evidence in support of the fact that Akt signaling inhibits AR activity (Lin *et al.*, 2001). To further characterize the modality of AR-PTEN (Akt) interaction, we took advantage of adenoviral constructs developed in our laboratories, to perform a number of experiments using PTEN positive (+) or negative (-) prostate cancer cell lines. Our data suggest that PTEN antagonizes AR transcriptional activity through inhibition of Akt activation and that this effect is not cell line or promoter-dependent. In addition, our data suggest that PTEN inhibition of AR

transcription does not depend on prevention of Akt-mediated AR phosphorylation. This suggests that the effect of PTEN on AR transcription is probably mediated by one of the downstream post-translational/transcriptional effects mediated by Akt. As PTEN is frequently inactivated in androgen independent prostate cancer, these results suggest that loss of PTEN function may facilitate activation of AR signaling and progression to androgen independence, and identifies the PTEN-Akt pathway as an additional therapeutic target for the treatment of androgen-independent prostate cancer.

MATERIAL AND METHODS.

Materials

Fetal bovine serum, tissue culture media and antibiotics were from Invitrogen Corporation (Carlsbad, CA). Chemicals were from Sigma (St. Louis, MO) unless stated otherwise. Restriction endonucleases were from New England Biolabs (Beverly, MA). Hybond ECL nitrocellulose membranes and ECL+plus Western Blotting Detection System were from Pharmacia Biotech (Piscataway, NJ) (Cat. # RPN303D and RPN2132, respectively). Antibodies were: PTEN (Cascade BioScience, Winchester, MA) (Cat. No. ABM-2052, working dilution:1000:1), Akt (total) (Cell Signaling, Beverly, MA) (Cat. No. # 9272, working dilution: 1000:1), (phospo)-Akt (^{473}Ser) (Cell Signaling, Cat. No. # 9271, working dilution: 1000:1), PSA (Dako, Carpinteria, CA) (Cat. # A0562, working dilution: 1000:1), β-Actin (Sigma, Cat. # A5441, working dilution: 5000:1), Bax (BD-Biosciences, Franklin Lakes, NJ) (Cat. # 554104, working dilution: 1000:1), AR (Santa Cruz Biotechnology, Santa Cruz, CA, Cat. # 816, working diluition 300:1). Secondary antibodies were: Anti-mouse IgG, peroxidase-linked (Amersham-Biotech, Piscataway NJ, Cat. # NA931, working dilution: 1000:1) and Anti-rabbit IgG, peroxidase-linked (Amersham-Biotech, Cat. # NA934, working dilution: 1000:1). Dihydrotestosterone was from Steraloids (Newport, RI, Cat. # A2571-000). R1881 was from NEN (Boston, MA). The PI3K inhibitor LY 294002 was from Cell Signaling (Cat. # 9901).

Plasmids

pCMV-AR contains the wt AR cDNA under the control of the CMV promoter (Tilley *et al.*, 1989). pCMV-AR S213A&S791A contains an AR cDNA in which the putative Akt phosphorylation sites S213 and S791 have been mutated from serine to

alanine residues. GRE_2E1b-Luc is a luciferase reporter plasmid driven by two androgen response elements from the tyrosine amino transferase promoter, followed by the adenovirus E1b TATA box (Allgood *et al.*, 1993). PRL-CMV-TK contains the Renilla luciferase cDNA (Promega, Madison WI) under the control of the constitutively active CMV promoter.

Adenoviral constructs

The following adenoviral constructs were used: Av-ARR_2PB-Bax, Av-CMV-PTEN, Av-CMV-PTEN(mut), Av-CMV, Adeno-myrAkt, Adeno-dnAkt, Av-CMV-GFP and Av-CMV-LacZ.

Preparation of adenovirus Av-ARR_2PB-Bax has already been described (Andriani *et al.*, 2001). This adenovirus contains a HA-tagged cDNA of the pro-apoptic protein Bax under the control of the ARR_2PB (Zhang *et al.*, 2000) promoter. The ARR_2PB promoter is inducible by AR only in AR (+) cell lines deriving from prostatic epthelium, after addition to the medium of dihydrotestosterone or non-metabolizable androgens such as mibolerone or R1881. The HA-Bax protein induced from this system after addition of androgens to the medium is recognizable from the wt form because is slightly larger by immunoblot analysis.

Adenoviruses Av-CMV-PTEN and Av-CMV-PTEN(mut) and Av-CMV have been previously described (Yuan and Whang, 2002). Av-CMV-PTEN contains the wild type PTEN cDNA under the control of the CMV promoter. Av-CMV-PTEN(mut) expresses a mutant form of PTEN (G129E), which has lost its lipid phosphatase activity and the ability to inhibit Akt activation (Yuan and Whang, 2002). Adenovirus Av-CMV contains the CMV promoter and no cDNA's subcloned downstream to it. Adenovirus

Av-CMV-LacZ has already been described, and contains the LacZ cDNA subcloned downstream of the CMV promoter (Marcelli *et al.*, 1999). Both Av-CMV and Av-CMV-LacZ were used as a control to Av-CMV-PTEN. The dominant-negative Akt mutant (Adeno-dnAkt) has alanine residues substituted for threonine at position 308 and serine at position 473 (Suhara *et al.*, 2001). The constitutively active Akt (Adeno-myrAkt) has the c-src myristoylation sequence fused in frame to the N-terminus of the wild-type Akt coding sequence that targets the fusion protein to the membrane. Membrane-bound Akt is constitutively active (Suhara *et al.*, 2001). The cDNA's of these Akt mutants were subcloned under the control of the CMV promoter, and inserted in the context of a replication-defective first generation adenovirus. Both these adenoviral constructs constructs were gifts of Dr. K. Walsh, Tuft University.

Adenovirus Av-CMV-GFP contains the cDNA of the green fluorescent protein under the control of the CMV promoter in the context of a first generation replication-defective adenovirus. This construct was a gift of M. Ittmann (Baylor College of Medicine), and was used to identify the ideal MOI for the various cell lines and as a negative control when required.

Cell Lines

Prostate cancer derived LNCaP (Horoszewicz *et al.*, 1980) (maintained in RPMI-1640, 10% FBS and 1% P&S), LNCaP-LP (maintained in RPMI 1649, 10% FBS and 1% P&S), LAPC-4 (Klein *et al.*, 1997) (provided by Dr. Charles Sawyers of UCLA, maintained in Iscove's Modified Dulbecco's Medium [Invitrogen, Cat. No. 12382-016], 15% FBS and 1% P&S) and PC-3 (Kaighn *et al.*, 1979) (maintained in F12 + 10% FBS + 1% P&S) were used for the experiments reported in this paper. LNCaP, LNCaP LP and

LAPC-4 were chosen because they contain the androgen receptor (AR), which is wild type in LAPC-4 (Klein *et al.*, 1997), and contains a well characterized (T877A) mutation in LNCaP and LNCaP LP cells (Veldscholdte *et al.*, 1990). The difference between LNCaP and LNCaP-LP cells is that while the former have been continuously passaged in our laboratory for the last 8 years, LNCaP-LP (low passage) were recently purchased from the ATCC (Manassas, VA), and used immediately after thawing in the experiments described below. PC-3 was chosen because this cell line is an example of an AR(-) (Tilley *et al.*, 1990) PTEN(-) (Li *et al.*, 1997) cell line of prostatic derivation.

Cell Proliferation assay

5 X 10^4 LNCaP cells were seeded per well in a 24-well plate and then infected with adenovirus Av-CMV or Av-CMV-PTEN (MOI 10) in media with charcoal stripped serum. 24 hours after infection, R1881 (0.05 nM) or vehicle was added to media. This dose of ligand was used because AR agonists are known to have a bifasic effect on LNCaP cells proliferation, consisting in a stimulatory activity at subsaturating doses, and an inhibitory activity at saturating doses (Lee *et al.*, 1995; Sonnenschein *et al.*, 1989; Zhao *et al.*, 1997). Cell proliferation was determined using the colorimetric MTT assay at 24 hour interval. Results shown are the MTT OD readings of triplicate wells expressed as the mean +/- SD and are representative of at least three independent experiments.

Measurement of PSA production by LNCaP cells

LNCaP cells (10^6 per well) were seeded in a 6-well plate. After 24 hours, cells were washed with phosphate-buffered saline and then incubated with 2 ml of serum-free medium. Then dihydrotesterone (2 nM) or LY294002 (10 or 20 μM) or vehicle as indicated was added to medium. After 24 hours, supernatant was collected and analyzed

for PSA by a commercially available ELISA assay (ICN Pharmaceutical, Costa Mesa, CA). PSA levels in the collected supernantant are expressed as ng/ml and represent the mean +/- SD of two independent experiments.

Experimental protocols

Two days before adenoviral infection, $1x10^5$ cells were seeded in each well of a six well plate. On the day of infection, cells from one well were detached with trypsin and counted. This information was used to infect each cell line at the desired multiplicity of infections (MOI). Infections were carried out with 500 μL of infection medium (the same medium used for each cell line with 2% FBS and 1% penicillin and streptomycin) in a 5% CO_2 incubator at 37 °C for 1 hour on a rocker. Pilot experiments with an adenovirus containing the green fluorescent protein (GFP) cDNA (Av-CMV-GFP) determined the optimal MOI for the cell lines used in this investigation. Based on this, LAPC-4 and LNCaP were infected with a MOI of 100:1 with every adenovirus used, except AvCMV-PTEN which was used at MOI of 1000:1 to achieve complete dephosphorylation of Akt in LNCaP cells.

These experiments were performed in regular fetal bovine serum (FBS). Use of regular FBS did not have consequences on AR trascriptional activation, as we have found that the concentrations of testosterone or dihydrotestosterone determined by radioimmunoassay in the FBS from Invitrogen are extremely low [17 ng/dl (59 pM), and 3 pg/ml (0.01 pM) for T and DHT, respectively] and unable to induce ARR_2PB activity under the experimental conditions used throughout these studies (data not shown)[1].

[1] Zhang Y., Marcelli M.: Manuscript in press in Human Gene Therapy

LNCaP, LNCaP-LP or LAPC-4 cells were infected with Av-CMV-PTEN, Av-CMV-PTEN(mut) or Av-CMV-LacZ on day 0. In some experiments adeno(dn)Akt or adeno(myr)Akt were infected simultaneously with the PTEN construct. After 48 hours cells were infected with Av-ARR$_2$PB-Bax and treated in the presence of vehicle alone or vehicle + DHT 2 nM. After 24 hours of hormonal stimulation cells were harvested and immunoblot analysis was performed for: PTEN (to control for successful infection with Av-PTEN or Av-PTEN-mut), total and ^{473}Ser-phospho-Akt (to control for *wt* PTEN activity, which is expected to prevent Akt phosphorylation, but not total Akt levels), β-actin (to control for equal loading in each lane), Bax to control for hormonal induction of AvARR$_2$-PB-Bax (the AR inducible construct in which Bax is controlled by the exogenous ARR$_2$PB promoter). In some experiment immunoblot analysis was performed for PSA (to control for DHT-induction of an endogenous AR-responsive gene). In each experiment the same number of μg of cell lysate was loaded. When precise quantitation was required, densitometric analysis was performed to correct expression of the protein of interest with that of β-actin, which was immunodetected in the same sample (Li *et al.*, 2001b). Densitometry was done by importing images to a Macintosh G4 personal computer using the Chemi Doc™ Documentation System, and the Quantity One quantitation software (both from BioRad, Hercules CA). Arbitrary densitometric units of the protein of interest were then corrected for the densitometric units of β-actin.

Site directed mutagenesis

S213 and S791are two putative Akt phosphorylation sites identified in the sequence of AR (Wen *et al.*, 2000). One possible mechanism used by PTEN to control AR transcriptional activation may be by modulating phosphorylation of these sites by

preventing Akt activation. To test this hypothesis, we performed site directed mutagenesis to obtain an AR cDNA in which S213 and S791 were changed into Alanine residues, using the QuickChange™ XL Site-Directed Mutagenesis kit (Stratagene, La Jolla CA). The procedure was performed according to the specifications of the manufacturer, using as a template the wt pCMV-AR expression plasmid, and primers $^{5'}$CGA GGG AGC GC**G** CGG GGG CTC CCA C$^{3'}$ and $^{5'}$GTG GGA GCC CCC G**C**G CGC TCC CTC G$^{3'}$ to obtain S213A-AR, and 5'TGA GGC ACC TC**G** CTC AAG AGT TTG G3' and 5'CCA AAC TCT TGA G**C**G AGG TGC CTC A^{3} to obtain S791A. Presence of the desired nucleotide substitutions and absence of unwanted PCR-related mutations in the resulting plasmids was confirmed by sequence analysis with a published sequence of wt AR (Tilley *et al.*, 1989). This analysis confirmed that S213 (*T*CG) and S791 (*T*CT) were mutagenized into alanine residues (*G*CG and *G*CT, respectively). The two resulting plasmids CMV-AR S213A and CMV-AR S791A were subsequently digested, and the two mutated fragments ligated to obtain plasmid CMV-AR S213A&S791A in which both mutations were correctly inserted within the same AR cDNA.

Transient transfections to evaluate the transcriptional activity of CMV-ARS213A&S791A

Non-recombinant adenoviral-mediated DNA transfer technique

To study if PTEN affects transcription of an AR cDNA mutated in its two putative Akt-phosphorylation sites, AR(-) and PTEN(-) PC-3 cells were infected with a virus encoding wt PTEN (or a control virus encoding LacZ) at MOI's of 5000:1. After 48 hours cells were transiently transfected with the non-recombinant adenoviral-mediated

DNA transfer technique (Allgood *et al.*, 1997) using 10 ng of CMV-AR, or CMV-AR S213A&S791A in association with 0.5 μg of the androgen-inducible GRE_2E1b-Luc reporter (expressing firefly luciferase activity upon induction), and 10 ng of the constitutively active pRL-CMV-TK plasmid (expressing renilla luciferase activity). Plasmids were incubated with the coupled virus (at a multiplicity of infection of 500:1) for 30 minutes. Subsequently, additional poly-L-lysine (1.3 μg/ μg of DNA) was added to shrink the DNA onto the viral surface. The virus-DNA complex was added to the cells and allowed to infect them for 2 hours in serum-free medium after which time the medium was supplemented with charcoal stripped serum to a final concentration of 5%. Each experiment was performed a minimum of three times.

Cell Treatment

Twenty-four hours after transfection, transfected PC-3 cells were treated with 2 nM DHT or 0.2% ethanol vehicle for 24 hours.

Western analysis for AR

Cell lysates from each well were divided into two aliquots. The first was used for the detection of luciferase activity (described below). Cell lysate volumes from the second aliquot equal to 10 μg of proteins were utilized for the immunodetection of AR and β-actin by Western analysis. Arbitrary densitometric units of the AR band of each well were corrected for the densitometric units of the corresponding β-actin band (AR/β actin DU ratio).

Luciferase activity

Luciferase activity was measured using the Dual-Luciferase® Reporter (DLRTM) Assay System (Promega, Madison Wisconsin). Results were expressed as LU/s (luciferase units

per second), and represent the ratio of the firefly (representing DHT-inducible luciferase activity from plasmid GRE_2E1b-Luc) and renilla (representing the constitutive luciferase activity from plasmid pRL-CMV-TK) luciferases activities detected in the cell lysate. Renilla luciferase activity was generated by a constitutively active plasmid and it was measured to correct firefly luciferase activity for differences of transfection efficiency among the various plates. The LU/s units obtained after this initial correction were further corrected for the AR/βactin DU ratio derived from the cell lysates of the same well, to normalize for differences in AR expression detected by the Western analysis step described above.

RESULTS

PI3K signaling inhibition prevents known effects of AR in LNCaP cells

PTEN(-) LNCaP cells were infected with adenoviral constructs Av-CMV-PTEN or Av-CMV and stimulated with R1881 (0.05 nM) or vehicle. Proliferation was significantly enhanced in Av-CMV infected cells after stimulation with R1881 in comparison with Av-CMV infected cells treated with vehicle alone. In contrast, R1881-stimulated proliferation was significantly inhibited in Av-CMV-PTEN infected cells (Fig. 1A). In additional experiments, we determined DHT-stimulated PSA concentration in the supernatant of LNCaP cells treated with 0, 10 or 20 μM of the PI3K inhibitor LY294002. Under these experimental conditions, DHT induced significant increase of PSA only in control cells, while increasing concentrations of LY294002 inhibited production of this surrogate marker of endogenous AR activation (Fig. 1B). These experiments suggested the possibility that inhibition of PI3K signaling may reduce AR activity. To rule out that reduced AR activity was due to decreased AR expression, quantitation of immunoreactive AR was performed during inhibition of PI3K signaling through LY294002 treatment or adenoviral-mediated PTEN re-expression, and no changes were detected compared to vehicle treated cells (Fig. 1C).

PTEN reduces AR transcriptional activity in a cell line and promoter-independent fashion

Further experiments were performed to determine how PTEN interferes with AR function. LNCaP cells were infected with Av-CMV-PTEN or Av-CMV-LacZ as a control, followed after 48 hours by infection with $AvARR_2PB$-Bax and treatment for 24 hours with 2 nM DHT. Western analysis of the resulting cell lysates are shown in Fig. 2.

Adenoviral-mediated expression of wt PTEN in high and low passage LNCaP cells inhibited Akt phosporylation, while no effect on total Akt expression was detected [Fig. 2A: compare lanes 1 and 2 (Av-CMV-LacZ infected) with 3 and 4 (Av-CMV-PTEN-infected), and lanes 5 and 6 (Av-CMV-LacZ infected) with 7 and 8 (Av-CMV-PTEN-infected) in LNCaP low and high passage, respectively]. Following infection with Av-ARR_2PB-Bax, treatment with DHT induced significant expression of HA-Bax in the absence of PTEN (recognizable by the appearance of the larger HA-Bax band in lanes 2 and 6 which were infected with the control virus Av-CMV-LacZ). However, when DHT was administered to LNCaP cells previously infected with Av-CMV-PTEN, induction of HA-Bax expression was significantly lower than in control cells infected with Av-CMV-LacZ [compare lanes 4 and 8 (infected with Av-CMV-PTENwt) with lanes 2 and 6 (infected with Av-CMV-LacZ)]. At least eight experiments were carried out looking at PTEN-induced inhibition of HA-Bax expression under these experimental conditions, and an average of 65% inhibition was seen. For instance the experiment of Fig. 2A (lanes 4 and 8 compared to 2 and 6, respectively) shows 100% inhibition, while the experiment of Fig. 2C (lane 2 compared to 4) shows 55% inhibition.

In addition to Av-CMV-LacZ, also the inactive lipid phosphatase deficient form of PTEN was used to control these experiments (Yuan and Whang, 2002). As shown in Fig 2C, despite its dramatic overexpression this form of PTEN was functionally inactive {shown by its inability to prevent Akt phosphorylation [compare lane 1 and 2 which were infected with Av-CMV-PTEN with lanes 3 and 4 which were infected with AvCMV-PTEN(mut)]}. HA-Bax expression from the androgen responsive virus AvARR_2PB-Bax was inhibited by the wild type (Fig. 2C lanes 1 and 2), but not mutant form of PTEN

(Fig. 2C, lanes 3 and 4), or the control virus Av-CMV-LacZ (Fig. 2C, lanes 5 and 6) following treatment with DHT.

PTEN-mediated inhibition of AR activity was also observed with PSA, an endogenous AR-regulated gene. The experiment of Fig. 2B showed that PSA expression is dramatically stimulated by DHT in LNCaP cells, and that this effect is prevented by adenoviral-mediated expression of wild type PTEN. This experiment was further controlled using the mutated PTEN adenovirus. Fig. 2C shows that PSA expression is inhibited by the wtPTEN, but not by the mutated PTEN or the control adenovirus Av-CMV-LacZ.

These experiments suggested therefore that in LNCaP cells wt PTEN antagonizes the ability of AR to induce expression of a reporter gene (HA-Bax) driven by an exogenous androgen-responsive promoter, and of a native gene (PSA) driven by an endogenous AR-responsive promoter. These experiments also demonstrated that the lipid phosphatase function of PTEN was required to achieve inhibition of DHT-induced expression of HA-Bax and PSA.

PTEN-mediated inhibition of AR activity is not cell line-dependent

We utilized AR(+) and PTEN (+) LAPC-4 cells to determine if the inhibitory effect of PTEN on AR activity is present in CaP cell lines other than LNCaP cells. The experiments of Fig. 3 show that adenoviral-mediated PTEN overexpression is associated in LAPC-4 cells with decreased DHT-dependent induction of HA-Bax, and PSA. Thus PTEN-induced antagonism of AR transcription is not cell line dependent.

PTEN-mediated inhibition of AR activity is Akt dependent

The fact that the lipid phosphatase activity of PTEN is necessary to antagonize Akt activation and AR transcription, led to the hypothesis that Akt is the mediator of the observed inhibitory effect of PTEN on AR transcription. Additional experiments were performed to demonstrate this point. LNCaP cells were again infected with Av-CMV-LacZ (MOI 100:1) or Av-CMV-PTEN (1000:1) for 48 hours, followed by Av-ARR_2PB-Bax (100:1) for 24 hours and treatment with 2 nM DHT for 24 hours. As shown in Figure 3, under control conditions (lanes 3 and 4) DHT induced significant amount of HA-Bax expression, while presence of wild type PTEN inhibited HA-Bax induction by 85% (lanes 5 and 6). In additional experiments, LNCaP cells were infected with Av-CMV-PTEN (MOI 1000:1) and Adeno-dnAkt (MOI 100:1) or Adeno-myrAkt (MOI 100:1) for 48 hours, followed by treatment for 24 hours with 2 nM DHT. Association of PTEN with the dominant negative Akt construct completely prevented HA-Bax expression (lanes 1 and 2), while association of wt PTEN with the constitutively active Akt construct (lanes 7 and 8) was able to revert (at least partially) the inhibitory effect of PTEN on HA-Bax expression observed in lanes 5 and 6. HA-Bax expression was rescued by 62% when LNCaP cells were infected with PTEN + myrAkt compared to PTEN alone. These experiments showed that a dominant negative form of Akt contributed with PTEN to inhibit AR transcriptional activity. In contrast, the constitutively active form of Akt antagonized this effect of PTEN. Together with the observation that the phosphatase deficient form of PTEN did not have an effect on Akt activation and AR transcription (Fig. 2C), these experiments supported the hypothesis that PTEN inhibits AR activity in an Akt-dependent way.

Does Akt modulate AR activity through its direct phosphorylation?

Investigators have reported that two putative Akt phosphorylation sites within the sequence of AR undergo Akt-mediated phosphorylation (Lin *et al.*, 2001; Wen *et al.*, 2000). We reasoned that if PTEN modulates AR transcription by inhibiting Akt activation, absence of these putative Akt phosphorylation sites should prevent inhibition of AR by PTEN. We performed site directed mutagenesis of these two putative phosphorylation sites to produce plasmid CMV-AR S213A&S791A, in which serine residues 213 and 791 are replaced by alanines. PC-3 cells were initially infected with Av-CMV-PTEN (or Av-CMV-LacZ as a control) for 48 hours, and subsequently transfected with pCMV-AR or pCMV-AR S213A&S791A and with reporter plasmids GRE_2E1b-Luc and PRL-CMV-TK. Vehicle or vehicle plus DHT were then added to the culture plates for 24 hours. This experiment showed that transcriptional activity of both AR plasmids was similarly inhibited by PTEN, therefore inhibition of AR transcriptional activity does not depend on prevention by PTEN of Akt-mediated AR phosphorylation, at least in the two putative sites mutated in this experiment and in the cell line PC-3.

DISCUSSION

This paper provides evidence in support of the hypothesis that in prostate cancer cell lines PTEN antagonizes AR transcriptional activity through inhibition of Akt activation, and that this effect is not cell line or promoter-dependent. Recent papers have suggested that two serines (S213 and S791) located in the midst of two Akt-consensus sites in the coding sequence of AR are phosphorylated by Akt, and that AR activity is affected by Akt-mediated phosphorylation. Based on this, we set an experiment to test the hypothesis that the observed ability of PTEN to antagonize AR transcription is due to direct Akt-mediated AR phosphorylation. An AR construct with alanine residues inserted in replacement of S213 and S791 was prepared and transfected in AR (-) and PTEN (-) PC-3 cells. PTEN exerted a similar inhibitory effect on transcriptional activity of both wt AR or AR S213A&S791A, suggesting that lack of the two putative Akt phosphorylation sites does not affect PTEN-mediated inhibition of AR transcription.

Akt regulates its target molecules by phosphorylation, and its activity results in survival, proliferation and cellular growth (Vivanco and Sawyers, 2002). Some of the activities resulting in survival consist in direct inactivation (by phosphorylation) of factors mediating cell death such as the apoptotic proteins Bad (Zha *et al.*, 1996) and Caspase-9 (Cardone *et al.*, 1998). In alternative, Akt-mediated phosphorylation stimulates survival by activating other factors such as Mdm2, a molecule whose ability to function as a survival factor depends on facilitating degradation of the pro-apoptotic tumor suppressor gene p53 (Mayo and Donner, 2001; Zhou *et al.*, 2001). A third mechanism through which Akt affects survival is by activating or inhibiting transcription factors responsible for the synthesis of antiapoptotic or proapoptotic genes, respectively.

For instance Akt indirectly (through phosphorylation of IκB) activates the transcription factor NF-κB (Romashkova and Makarov, 1999), which affects survival by transcribing the antiapoptotic genes TRAF1, TRAF2, c-IAP1, cIAP2 and c-FLIP (Micheau *et al.*, 2001; Wang *et al.*, 1998). An example of a transcription factor responsible for the transcription of pro-apoptotic molecules such as FAS ligand (Brunet *et al.*, 1999) and BIM (Dijkers *et al.*, 2000) is the Forkhead transcription factor FHKR. Akt inhibits FHKR by anchoring it to the cytosol through phsphorylation (Brunet *et al.*, 1999). In addition to regulating cell survival, Akt also induces cellular proliferation and growth by phosphorylating a variety of substrates using the same general mechanisms (Vivanco and Sawyers, 2002). Based on this, one can conclude that PTEN-mediated inhibition of Akt activation has several potential ways to affect AR transcription. Akt could affect AR transcription by post translationally modifying substrates required for AR activation or repression, or in alternative could modulate in a positive or negative way the transcription of such factors. Identification of these Akt-regulated regulators of AR transcriptional activity is one of the projects currently going on in or laboratory.

A number of studies have recently examined the interaction existing between AR and PTEN/Akt signaling, and the conclusions are controversial. Wen and collaborators were the first to identify the presence of two Akt consensus sites in AR in Ser213 and 791, and to show that Akt can directly bind to and phosphorylate AR (Wen *et al.*, 2000). Lin et al (Lin *et al.*, 2001) demonstrated Akt-mediated AR phosphorylation in Ser213 and 791. These authors also described that active Akt inhibits AR transcriptional activity, and that this effect is mimicked by the constitutively active form of Akt, and inhibited by the dominant negative Akt construct. According to these authors, inhibition of AR

activity goes through two steps, a first step of Akt-mediated phosphorylation and a second step of Mdm2-mediated ubiquitination (Lin *et al.*, 2002). The reasons for the discrepancy between ours and their data is not clear, but has probably to do with the fact that we used different cell lines (LNCaP and LAPC-4 instead of DU-145). Additionally, we used assays measuring the mitogenic and anti-apoptotic effects of AR, while Lin et al used a model of AR-induced apoptosis.

Posttranslational modifications of AR such as phosphorylation have been suggested to be an important mechanism modulating AR activity for a number of years (Blok *et al.*, 1996; Kemppainen *et al.*, 1992). Using a combination of peptide mapping, Edman degradation, and mass spectrometry (Gioeli *et al.*, 2002), Gioeli et al have mapped the phosphorylation sites of AR, which do not include S213 and S 791, possibly due to the non-selectivity of the in vitro kinase reactions which were utilized to identify these two sites. In agreement with the data of Gioeli, we did not find differences in the transcriptional activity of CMV-AR and CMV-AR S213A&S791A and detected similar degrees of suppression when the experiments were done in the presence of PTEN, suggesting that these two phosphorylation sites are not used by Akt to modulate AR activity, at least in PC-3 cells.

The negative interaction between inhibition of the PI3K pathway and AR transcriptional activity described in this paper is supported in the literature by the papers of Li (Li *et al.*, 2001a) and Sharma (Sharma *et al.*, 2002). Similarly to us, Li and collaborators found that PTEN antagonizes AR signaling, and that this occurs in an Akt-dependent way. Sharma et al. not only described a negative interaction between PI3K

inhibition and AR signaling, but also that this is mediated by downregulation of β-catenin, an AR co-activator (Truica *et al.*, 2000).

In conclusion, our studies support the theory that PTEN functions as a transcriptional inhibitor of AR by preventing Akt activation, and that a downstream effect of the protein kinase Akt mediates this interaction. Unchecked Akt activation, which is frequently observed in advanced prostate cancer, may be associated with uncontrolled AR signaling, which may explain why androgen independent prostate cancer cells are insensitive to hormonal manipulation, but still require AR for their survival/proliferation. Furthering our knowledge on the PTEN (Akt)-AR axis will most likely create new therapeutic targets for androgen-independent prostate cancer.

ACKNOWLEDGEMENTS

We thank Dr. C. Sawyers (UCLA), M. Ittmann (Baylor), K. Walsh (Tuft) for some of the reagents utilized in the experiments described in this paper.

Supported by grants from the Department of Defense Prostate Cancer Research Program (DAMD17-00-1-0037) and NIH (CA85772) to Y.E.W., and Department of Defense Prostate Cancer Research (DAMD17-00-1-0075) and Merit Review Program of the Veterans Administration to M.M.

LEGENDS

Fig. 1: Inhibition of the PI3K pathway prevents established functions of AR. A: LNCaP cells were infected with adenoviral construct Av-CMV-PTEN (wt) or with the empty control Av-CMV and grown in the presence of vehicle or R1881 (0.05 nM). At 24 hr interval, the relative cell growth was determined by the MTT assay. Data are expressed as MTT OD, and represent the mean ± SD of triplicate wells. B: LNCaP cells were incubated in the presence of DHT (2 nM) or vehicle, and subjected to further treatment with LY294002 20 μM, 10 μM or vehicle. The amount of PSA in the culture supernatant was determined by an ELISA assay from aliquots of the supernatant obtained after 24 hours of treatment. Data represents the mean ± SD of two independent experiments. C: LNCaP cells were treated with the PI3K inhibitor LY 294002 (20 μM) (lanes 1 and 2) vehicle alone (lanes 5 and 6) or were infected with Av-CMV-PTEN (lanes 3 and 4) for 24 or 48 hours. Lysates from each experiment were subjected to western analysis for the immunodetection of AR and β-actin. The ratio of the AR/β-actin densitometric units did not show any difference in the six lanes of the experiment (not shown). Data represent a representative image of three independent experiments.

Fig. 2: PTEN inhibits expression of exogenous and endogenous AR-regulated genes. A: LNCaP cells (high and low passage clones) were infected with MOI 1000:1 of adenovirus Av-CMV-PTEN (WT) or Av-CMV-LacZ on day 0. After 48 hours cells were infected with $AvARR_2PB$-Bax (MOI 100:1) and incubated with DHT or vehicle for additional 24 hours. Immunoblot analysis was performed for β-actin (to control for equal

loading), p-Akt, total Akt, PTEN and Bax (the gene placed under the control of the AR-responsive promoter ARR_2PB). Note that exogenous Bax contains a HA tag, and runs slightly higher than endogenous Bax. B: LNCaP cells were infected with Av-CMV-PTEN (lanes 1, 2) or a control adenovirus Av-CMV-LacZ (lanes 3, 4) and treated with 2 nM DHT or vehicle for 24 hours. Immunoblot analysis was done for PTEN, β-actin (to control for equal loading), and PSA. The experiment of panel B was performed with the same cell lysates of panel A. C: LNCaP (high passage) were infected with Av-CMV-PTEN, Av-CMV-PTEN(mut) or Av-CMV-LacZ (MOI 1000:1) at time point 0. After 48 hours cells were infected with Av-ARR_2PB-Bax and incubated with 2 nM DHT or vehicle for the following 24 hours. Cell lysates were utilized to perform Western analysis of β-actin, total Akt, p-Akt, PTEN, Bax and PSA. The data suggest that wt (lanes 1 and 2) but not mutant PTEN (lanes 3 and 4) or the control adenovirus Av-CMV-LacZ (lanes 5 and 6) inhibits DHT-mediated expression of the exogenous (Bax) and endogenous (PSA) AR-dependent genes.

Fig. 3: PTEN inhibits the AR-induced expression of endogenous (PSA) and exogenous (HA-Bax) AR-dependent genes in the cell line LAPC-4. Cells were seeded on day 0. After 24 hours cells were infected with adenovirus Av-CMV-PTEN or Av-CMV-LacZ (MOI 100:1). After 48 hours cells were infected with adenovirus Av-ARR_2PB-Bax (MOI 100:1), followed by incubation in the presence of DHT or vehicle for additional 24 hours. Cell lysates were subjected to western analysis for β-actin (to control for equal loading), Bax (the gene placed under the control of the AR-responsive promoter

ARR_2PB), PTEN, total Akt, p-Akt, and PSA (the endogenous AR-responsive gene). Note that the endogenous level of Bax in this cell line is much lower than in LNCaP, and that a band migrating below HA-Bax is visible only in lane 2. The data suggest that DHT-mediated induction of Bax and PSA is inhibited in the cells infected with the PTEN virus [compare lane 2 (addition of DHT and absence of PTEN) with lane 4(addition of DHT and PTEN)].

Figure 4: A constitutively active Akt construct antagonizes PTEN while a dominant negative Akt construct has an additive effect on DHT-induced Bax expression from the Av-ARR_2PB-Bax construct. LNCaP cells were seeded on day 0, infected with Av-CMV-PTEN (lanes 1, 2, 5, 6, 7, and 8) or with the control adenovirus Av-CMV-LacZ (lanes 3 and 4) on day 1 (MOI 1000:1). Some wells were also infected with Adeno-dnAkt (lanes 1 and 2) or Adeno-myrAkt (lanes 7 and 8) viruses (MOI 100:1). After 48 hours cells were infected with Av-ARR_2PB-Bax (MOI 100:1), followed by incubation with DHT for 24 hours. Under control conditions (lanes 3 and 4) DHT induced large amount of HA-Bax from construct ARR_2PB-Bax. Bax induction was reduced in the presence of wt PTEN (lanes 5 and 6). This effect was antagonized in the presence of myrAkt, and further stimulated in the presence of dnAkt.

Fig. 5: PTEN similarly inhibits DHT-induced luciferase activity from PC-3 cells transfected with a wt AR construct or an AR construct with mutagenized Ser213 and 791.

Cells were infected with a control virus (Av-CMV-LacZ), or with Av-CMV-PTEN on day 0. After 48 hours cells were transfected with pCMV-AR (wtAR in the figure) or pCMV-AR S213A&S791A (mutAR in the figure), the reporter plasmid GRE2E1b-Luc, and the constitutively active plasmid PRL-CMV-TK expressing Renilla luciferase. Cells were then incubated with 2 nM DHT or vehicle for additional 24 hours. Luciferase activity was determined and corrected for transfection efficiency and for AR expression as described under "Material and Methods". Data are compared to cells infected with Av-CMV-LacZ + wtAR + DHT set at 100% and represents mean±SD of six wells. One of three experiments is shown in the picture.

REFERENCES

Allgood, V. E., Oakley, R. H., and Cidlowski, J. A. 1993 Modulation by vitamin B6 of glucocorticoid receptor-mediated gene expression requires transcription factors in addition to the glucocorticoid receptor. *J Biol Chem* **268**, 20870-20876.

Allgood, V. E., Zhang, Y., O'Malley, B. W., and Weigel, N. L. 1997 Analysis of chicken progesterone receptor function and phosphorylation using an adenovirus-mediated procedure for high-efficiency DNA transfer. *Biochemistry* **36**, 224-232.

Andjelkovic, M., Alessi, D. R., Meier, R., Fernandez, A., Lamb, N. J., Frech, M., Cron, P., Cohen, P., Lucocq, J. M., and Hemmings, B. A. 1997 Role of translocation in the activation and function of protein kinase B. *J Biol Chem* **272**, 31515-31524.

Andriani, F., Nan, B., Yu, J., Li, X., Weigel, N. L., McPhaul, M. J., Kasper, S., Kagawa, S., Fang, B., Matusik, R. J., Denner, L., and Marcelli, M. 2001 Use of the probasin promoter ARR(2)PB to express bax in androgen receptor-positive prostate cancer cells. *J Natl Cancer Inst* **93**, 1314-1324.

Balk, S. P. 2002 Androgen receptor as a target in androgen-independent prostate cancer. *Urology* **60**, 132-138.

Blok, L. J., de Ruiter, P. E., and Brinkmann, A. O. 1996 Androgen receptor phosphorylation. *Endocr Res* **22**, 197-219.

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. 1999 Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857-868.

Cantley, L. C. 2002 The phosphoinositide 3-kinase pathway. *Science* **296**, 1655-1657.

Cardone, M. H., Roy, N., Stennicke, H. R., Salvesen, G. S., Franke, T. F., Stanbridge, E., Frisch, S., and Reed, J. C. 1998 Regulation of cell death protease caspase-9 by phosphorylation. *Science* **282**, 1318-1321.

Craft, N., Shostak, Y., Carey, M., and Sawyers, C. 1999 A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. *Nature Med* **5**, 280-285.

Culig, Z., Hobish, A., Cronauer, M. V., Radmayr, C., Trapman, J., Hittmair, A., Bartsch, G., and Klocker, H. 1994 Androgen receptor activation in prostatic tumor cell lines by insulin-like growth factor I, Keratinocyte growth factor, and epidermal growth factor. *Cancer Res* **54**, 5474-5478.

Denmeade, S. R., Lin, X. S., and Isaacs, J. T. 1996 Role of programmed (apoptotic) cell death during the progression and therapy for prostate cancer. *Prostate* **28**, 251-265.

Dijkers, P. F., Medema, R. H., Lammers, J. W., Koenderman, L., and Coffer, P. J. 2000 Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol* **10**, 1201-1204.

Dong, J. T., Li, C. L., Sipe, T. W., and Frierson, H. F., Jr. 2001 Mutations of PTEN/MMAC1 in primary prostate cancers from Chinese patients. *Clin Cancer Res* **7**, 304-308.

Eisenberger, M. A., Blumenstein, B. A., Crawford, E. D., Miller, G., McLeod, D. G., Loehrer, P. J., Wilding, G., Sears, K., Culkin, D. J., Thompson, I. M., Jr., Bueschen, A. J., and Lowe, B. A. 1998 Bilateral orchiectomy with or without flutamide for metastatic prostate cancer. *N Engl J Med* **339**, 1036-1042.

Gioeli, D., Ficarro, S. B., Kwiek, J. J., Aaronson, D., Hancock, M., Catling, A. D., White, F. M., Christian, R. E., Settlage, R. E., Shabanowitz, J., Hunt, D. F., and Weber, M. J. 2002 Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites. *J Biol Chem* **277**, 29304-29314.

Hankey, B. F., Feuer, E. J., Clegg, L. X., Hayes, R. B., Legler, J. M., Prorok, P. C., Ries, L. A., Merrill, R. M., and Kaplan, R. S. 1999 Cancer surveillance series: interpreting trends in prostate cancer-- part I: Evidence of the effects of screening in recent prostate cancer incidence, mortality, and survival rates. *J Natl Cancer Inst* **91**, 1017-1024.

Horoszewicz, J. S., Leong, S. S., Chu, T. M., Wajsman, Z. L., Friedman, M., Papsidero, L., Kim, U., Chai, L. S., Kakati, S., Arya, S. K., and Sandberg, A. A. 1980 The LNCaP cell line--a new model for studies on human prostatic carcinoma. *Prog Clin Biol Res* **37**, 115-132.

Jemal, A., Thomas, A., Murray, T., and Thun, M. 2002 Cancer statistics, 2002. *CA Cancer J Clin* **52**, 23-47.

Kaighn, M. E., Narayan, K. S., Ohnuki, Y., Lechner, J. F., and Jones, L. W. 1979 Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest Urol* **17**, 16-23.

Kemppainen, J. A., Lane, M. V., Sar, M., and Wilson, E. M. 1992 Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation. Specificity for steroids and antihormones. *J Biol Chem* **267**, 968-974.

Klein, K. A., Reiter, R. E., Redula, J., Moradi, H., Zhu, X. L., Brothman, A. R., Lamb, D. J., Marcelli, M., Belldegrun, A., Witte, O. N., and Sawyers, C. L. 1997 Progression of metastatic human prostate cancer to androgen independence in immunodeficient SCID mice. *Nat Med* **3**, 402-408.

Lee, C., Sutkowski, D., Sensibar, J., Zelner, D., Kim, I., Amsel, I., Shaw, N., Prins, G., and Kozlowski, J. 1995 Regulation of proliferation and production of prostate specific antigen in androgen-sensitive prostate cancer cells, LNCaP, by dihydrotestosterone. *Endocrinology* **136**, 796-803.

Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M. H., and Parsons, R. 1997 PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* **275**, 1943-1947.

Li, P., Nicosia, S. V., and Bai, W. 2001a Antagonism between PTEN/MMAC1/TEP-1 and androgen receptor in growth and apoptosis of prostatic cancer cells. *J Biol Chem* **276**, 20444-20450.

Li, X.-Y., Marani, M., Mannucci, R., Kinsey, B., Andriani, F., Nicoletti, I., Denner, L., and Marcelli, M. 2001b Overexpression of BCL-X_L underlies the molecular basis for resistance to staurosporine-induced apoptosis on PC-3 cells. *Can Res* **61**, 1699-1706.

Lin, H. K., Wang, L., Hu, Y. C., Altuwaijri, S., and Chang, C. 2002 Phosphorylation-dependent ubiquitylation and degradation of androgen receptor by Akt require Mdm2 E3 ligase. *Embo J* **21**, 4037-4048.

Lin, H. K., Yeh, S., Kang, H. Y., and Chang, C. 2001 Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor. *Proc Natl Acad Sci U S A* **98**, 7200-7205.

Marcelli, M., Cunningham, G., Walkup, M., He, Z., Sturgis, L., Kagan, C., Mannucci, R., Nicoletti, I., Teng, B., and Denner, L. 1999 Signaling pathway activated during apoptosis of the prostate cancer cell line LNCaP: overexpression of caspase-7 as a new gene therapy strategy for the treatment of prostate cancer. *Cancer Res* **59**, 398-406.

Marcelli, M., Ittmann, M., Mariani, M., Sutherland, R., Nigam, R., Murthy, L., Zhou, Y., DiConcini, D., Puxeddu, E., Esen, A., Eastham, J., Weigel, N. L., and Lamb, D. J. 2000 Androgen receptor mutations in prostate cancer. *Can Res* **60**, 944-949.

Mayo, L. D., and Donner, D. B. 2001 A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus. *Proc Natl Acad Sci U S A* **98**, 11598-11603.

McMenamin, M. E., Soung, P., Perera, S., Kaplan, I., Loda, M., and Sellers, W. R. 1999 Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage. *Cancer Res* **59**, 4291-4296.

Micheau, O., Lens, S., Gaide, O., Alevizopoulos, K., and Tschopp, J. 2001 NF-kappaB signals induce the expression of c-FLIP. *Mol Cell Biol* **21**, 5299-5305.

Nazareth, L., and Weigel, N. 1996 Activation of the human androgen receptor through a protein kinase A signaling pathway. *J Biol Chem* **271**, 19900-19907.

Oliver, S. E., May, M. T., and Gunnell, D. 2001 International trends in prostate-cancer mortality in the "PSA ERA". *Int J Cancer* **92**, 893-898.

Romashkova, J. A., and Makarov, S. S. 1999 NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* **401**, 86-90.

Schroder, F. H. 1999 Endocrine treatment of prostate cancer - recent developments and the future. Part 1: maximal androgen blockade, early vs delayed endocrine treatment and side-effects. *BJU Int* **83**, 161-170.

Sharma, M., Chuang, W. W., and Sun, Z. 2002 Phosphatidylinositol 3-kinase/Akt stimulates androgen pathway through GSK3beta inhibition and nuclear beta-catenin accumulation. *J Biol Chem* **277**, 30935-30941.

Sonnenschein, C., Olea, N., Pasanen, M., and Soto, A. 1989 Negative controls of cell proliferation: human prostate cancers and androgens. *Cancer Res* **49**, 3474-3481.

Stocker, H., Andjelkovic, M., Oldham, S., Laffargue, M., Wymann, M. P., Hemmings, B. A., and Hafen, E. 2002 Living with Lethal PIP3 Levels: Viability of Flies Lacking PTEN Restored by a PH Domain Mutation in Akt/PKB. *Science* **295**, 2088-2091.

Suhara, T., Mano, T., Oliveira, B. E., and Walsh, K. 2001 Phosphatidylinositol 3-kinase/Akt signaling controls endothelial cell sensitivity to Fas-mediated apoptosis via regulation of FLICE- inhibitory protein (FLIP). *Circ Res* **89**, 13-19.

Suzuki, H., Freije, D., Nusskern, D. R., Okami, K., Cairns, P., Sidransky, D., Isaacs, W. B., and Bova, G. S. 1998 Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues. *Cancer Res* **58**, 204-209.

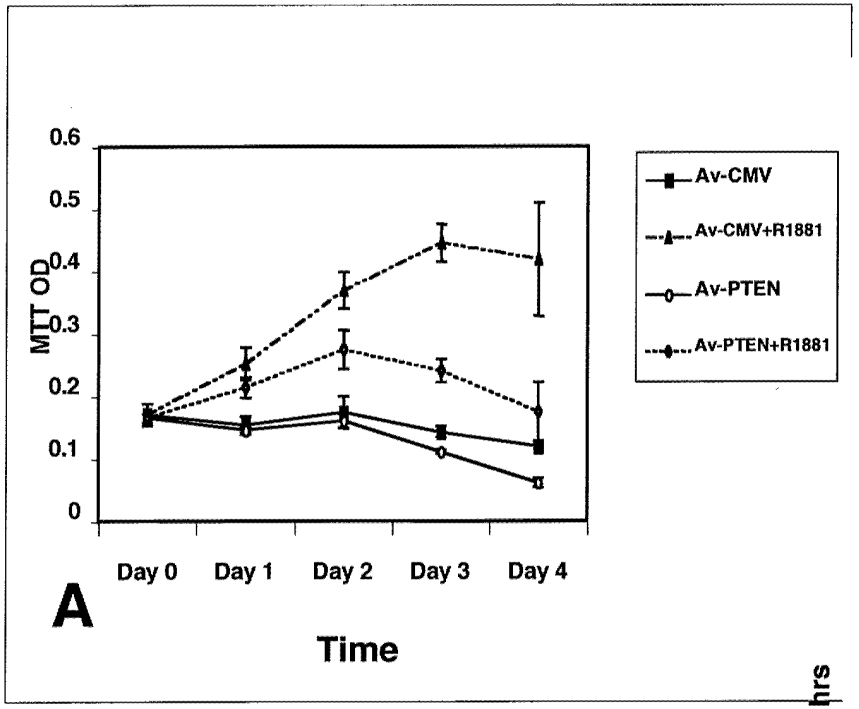
Taplin, M. E., Bubley, G. J., Ko, Y. J., Small, E. J., Upton, M., Rajeshkumar, B., and Balk, S. P. 1999 Selection for androgen receptor mutations in prostate cancers treated with androgen antagonist. *Cancer Res* **59**, 2511-2515.

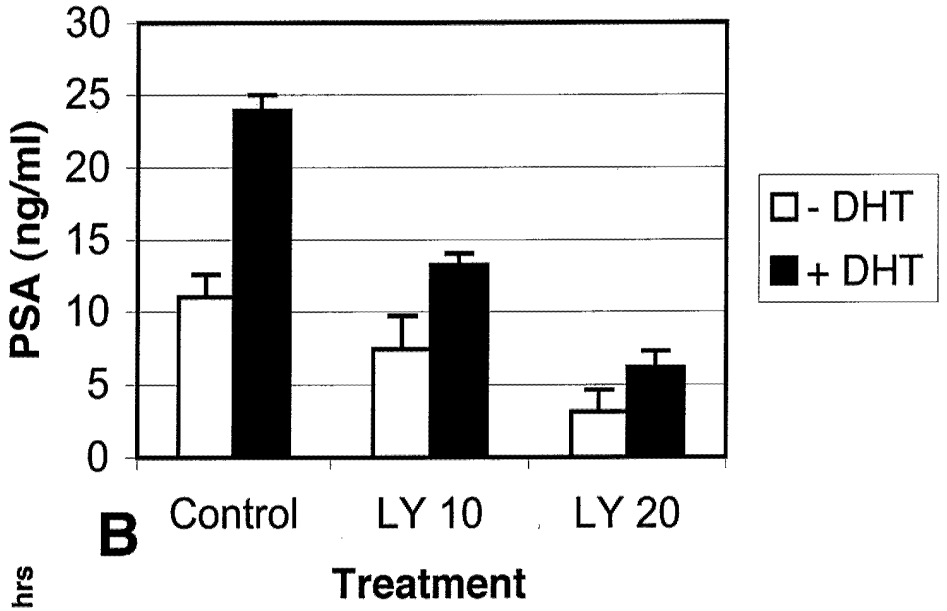
Tilley, W. D., Marcelli, M., Wilson, J. D., and McPhaul, J. M. 1989 Characterization and cloning of a cDNA encoding the human androgen receptor. *Proc Natl Aca Sci USA* **86**, 327-331.

Tilley, W. D., Wilson, C. M., Marcelli, M., and McPhaul, M. J. 1990 Androgen receptor gene expression in human prostate carcinoma cell lines. *Cancer Res* **50**, 5382-5386.

Truica, C. I., Byers, S., and Gelmann, E. P. 2000 Beta-catenin affects androgen receptor transcriptional activity and ligand specificity. *Cancer Res* **60**, 4709-4713.
Ueda, T., Bruchovsky, N., and Sadar, M. D. 2002a Activation of the androgen receptor N-terminal domain by interleukin-6 via MAPK and STAT3 signal transduction pathways. *J Biol Chem* **277**, 7076-7085.
Ueda, T., Mawji, N. R., Bruchovsky, N., and Sadar, M. D. 2002b Ligand-independent activation of the androgen receptor by IL-6 and the role of the coactivator SRC-1 in prostate cancer cells. *J Biol Chem*.
Van-der-Kwast, T. H., Schalken, J., Ruizeveld-de-Winter, J. A., Van-Vroonhoven, C. C. J., Mulder, E., Boersma, W., and Trapman, J. 1991 Androgen receptors in endocrine-therapy resistant human prostate cancer. *Int J Can* **48**, 189-193.
Vanhaesebroeck, B., and Alessi, D. R. 2000 The PI3K-PDK1 connection: more than just a road to PKB. *Biochem J* **346 Pt 3**, 561-576.
Veldscholdte, J., Ris-Stalpers, C., Kuiper, G. G. J. M., Jentser, G., Berrevoets, C., Claassen, E., Rooij, H. C. J. V., Trapman, J., Brinkmann, A. O., and Mulder, E. 1990 A mutation in the ligand binding domain of the androgen receptor of LnCAP cells affects steroid binding characteristics and response to anti-androgens. *Biochem Biophys Res Commun* **173**, 534-540.
Visakorpi, T., Hyytinen, E., Koivisto, P., Tanner, M., Keinanen, R., Palmberg, C., Palotie, A., Tammela, T., Isola, J., and Kallioniemi, O. P. 1995 In vivo amplification of the androgen receptor gene and progression of human prostate cancer. *Nat Genet* **9**, 401-406.
Vivanco, I., and Sawyers, C. L. 2002 The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer* **2**, 489-501.
Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. 1998 NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* **281**, 1680-1683.
Wen, Y., Hu, M. C., Makino, K., Spohn, B., Bartholomeusz, G., Yan, D. H., and Hung, M. C. 2000 HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. *Cancer Res* **60**, 6841-6845.
Yeh, S., Lin, H. K., Kang, H. Y., Thin, T. H., Lin, M. F., and Chang, C. 1999 From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. *Proc Natl Acad Sci U S A* **96**, 5458-5463.
Yuan, X. J., and Whang, Y. E. 2002 PTEN sensitizes prostate cancer cells to death receptor-mediated and drug-induced apoptosis through a FADD-dependent pathway. *Oncogene* **21**, 319-327.
Zegarra-Moro, O. L., Schmidt, L. J., Huang, H., and Tindall, D. J. 2002 Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. *Cancer Res* **62**, 1008-1013.
Zha, J., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S. 1996 Serine phosphorilation of death agonist BAD in response to survival factor results in binding to 14-3-3 not Bcl-xl. *Cell* **87**, 619-628.
Zhang, J. F., Thomas, T. Z., Kasper, S., and Matusik, R. J. 2000 A small composite probasin promoter confers high levels of prostate-specific gene expression through

regulation by androgens and glucocorticoids *in vitro* and i*n vivo*. *Endocrinology* **141**, 4698-4710.
Zhao, X. Y., Ly, L. H., Peehl, D. M., and Feldman, D. 1997 1alpha,25-dihydroxyvitamin D3 actions in LNCaP human prostate cancer cells are androgen-dependent. *Endocrinology* **138**, 3290-3298.
Zhou, B. P., Liao, Y., Xia, W., Zou, Y., Spohn, B., and Hung, M. C. 2001 HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. *Nat Cell Biol* **3**, 973-982.


0.6
0.5
0.4
0.3
0.2
0.1
0
MTT OD
Day 0
Day 1
Day 2
Day 3
Day 4
Time
A
Av-CMV
Av-CMV+R1881
Av-PTEN
Av-PTEN+R1881


30
25
20
15
10
5
0
PSA (ng/ml)
Control
LY 10
LY 20
Treatment
B
- DHT
+ DHT

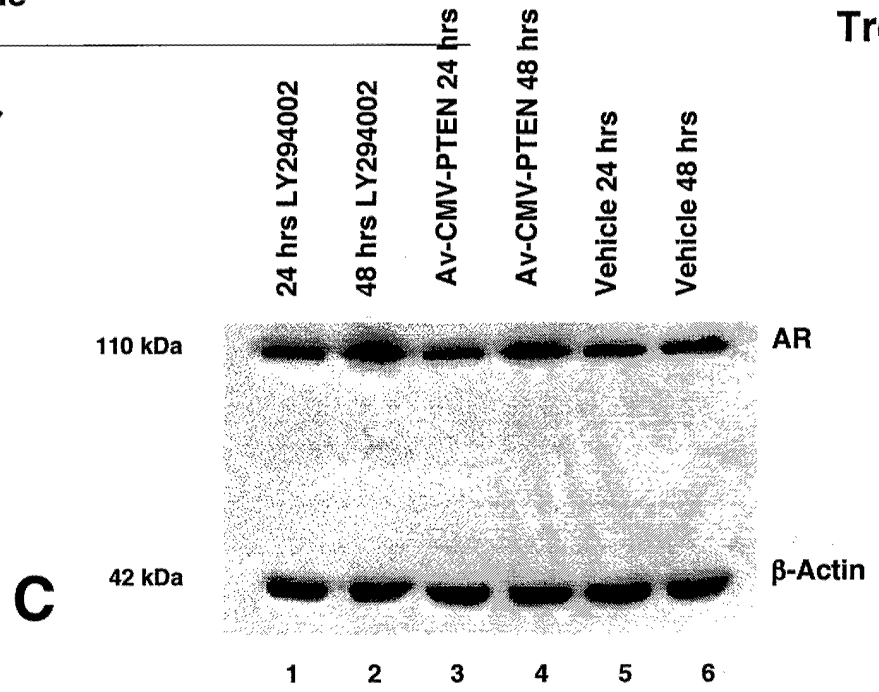

24 hrs LY294002
48 hrs LY294002
Av-CMV-PTEN 24 hrs
Av-CMV-PTEN 48 hrs
Vehicle 24 hrs
Vehicle 48 hrs
110 kDa
AR
42 kDa
β-Actin
1
2
3
4
5
6
C

Figure 1

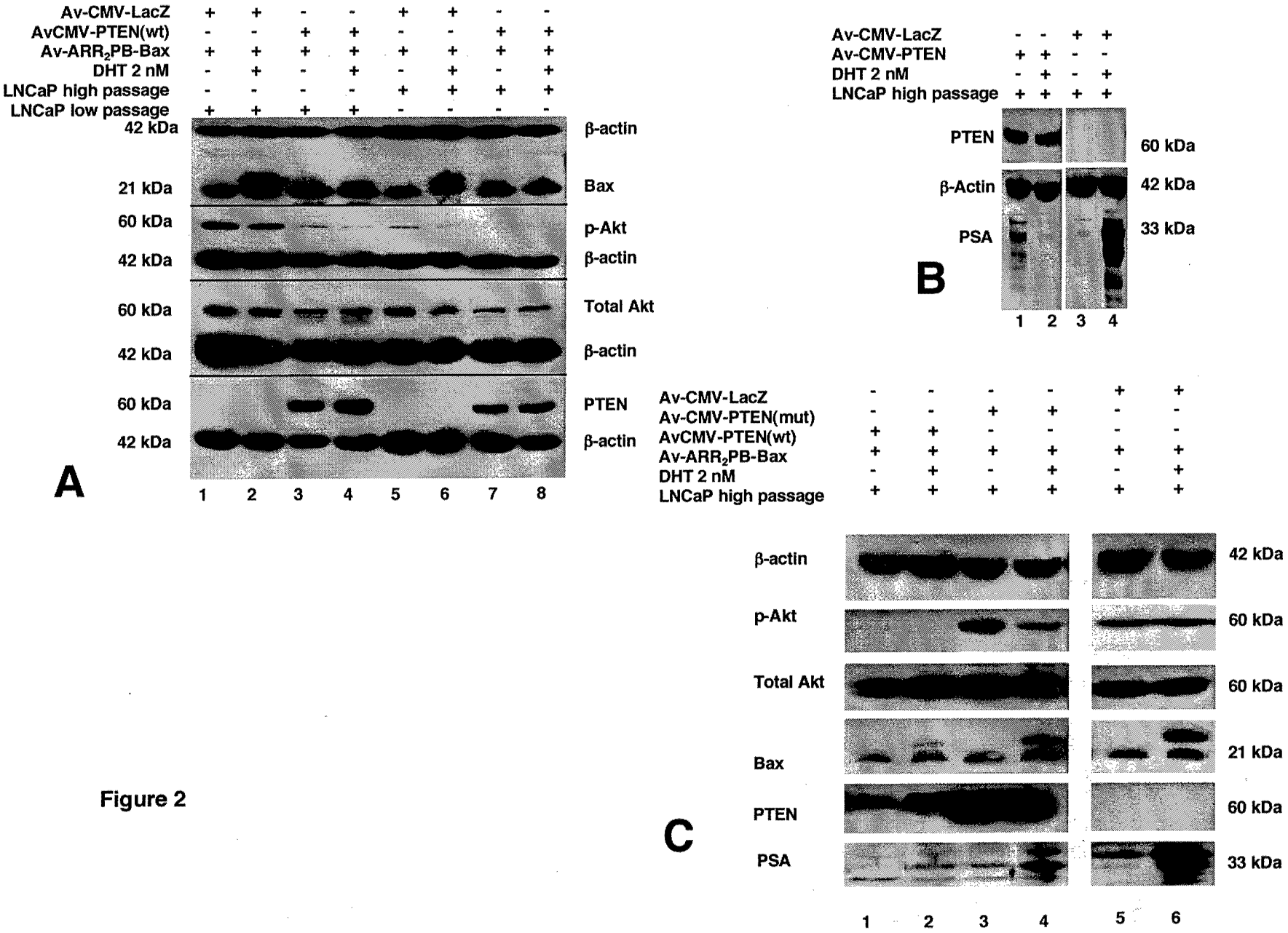

Av-CMV-LacZ + + - - + + - -
AvCMV-PTEN(wt) - - + + - - + +
Av-ARR2PB-Bax + + + + + + + +
DHT 2 nM - + - + - + - +
LNCaP high passage - - - - + + + +
LNCaP low passage + + + + - - - -
42 kDa
β-actin
21 kDa
Bax
60 kDa
p-Akt
42 kDa
β-actin
60 kDa
Total Akt
42 kDa
β-actin
60 kDa
PTEN
42 kDa
β-actin
A
1 2 3 4 5 6 7 8
Av-CMV-LacZ - - + +
Av-CMV-PTEN + + - -
DHT 2 nM - + - +
LNCaP high passage + + + +
PTEN
60 kDa
β-Actin
42 kDa
PSA
33 kDa
B
1 2 3 4
Av-CMV-LacZ - - - - + +
Av-CMV-PTEN(mut) - - + + - -
AvCMV-PTEN(wt) + + - - - -
Av-ARR2PB-Bax + + + + + +
DHT 2 nM - + - + - +
LNCaP high passage + + + + + +
β-actin
42 kDa
p-Akt
60 kDa
Total Akt
60 kDa
Bax
21 kDa
PTEN
60 kDa
PSA
33 kDa
C
1 2 3 4 5 6

Figure 2

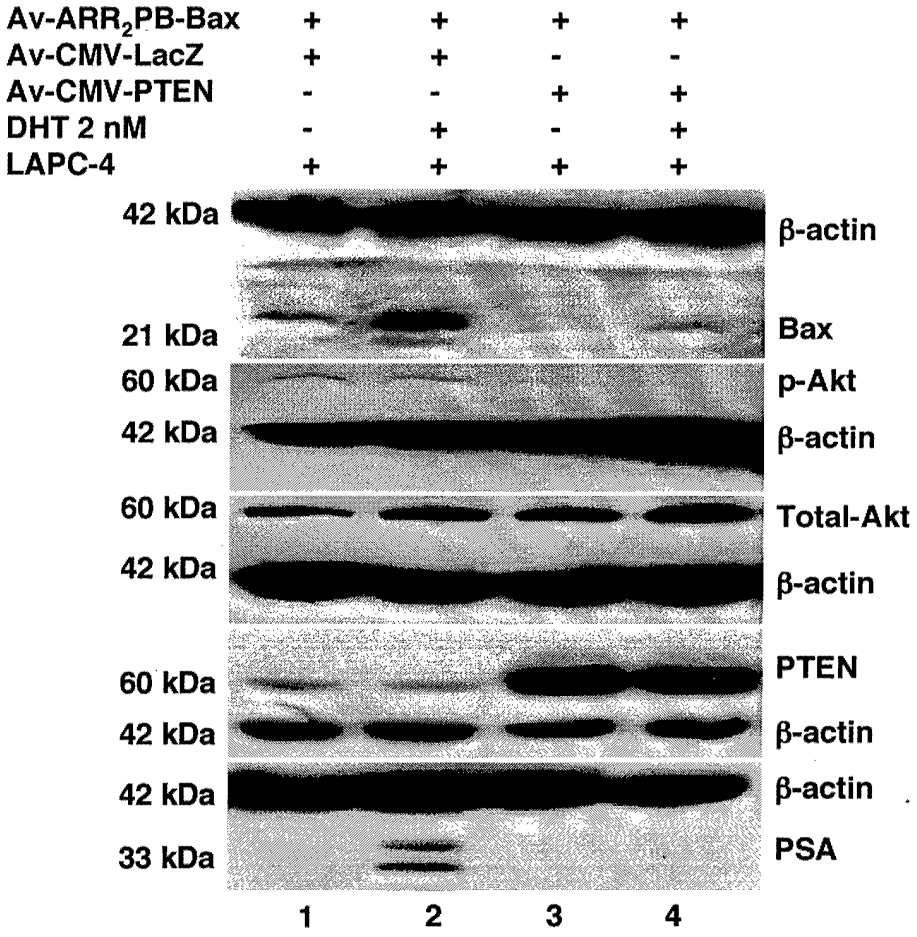

Av-ARR2PB-Bax + + + +
Av-CMV-LacZ + + - -
Av-CMV-PTEN - - + +
DHT 2 nM - + - +
LAPC-4 + + + +
42 kDa
β-actin
21 kDa
Bax
60 kDa
p-Akt
42 kDa
β-actin
60 kDa
Total-Akt
42 kDa
β-actin
60 kDa
PTEN
42 kDa
β-actin
42 kDa
β-actin
33 kDa
PSA
1 2 3 4

Figure 3

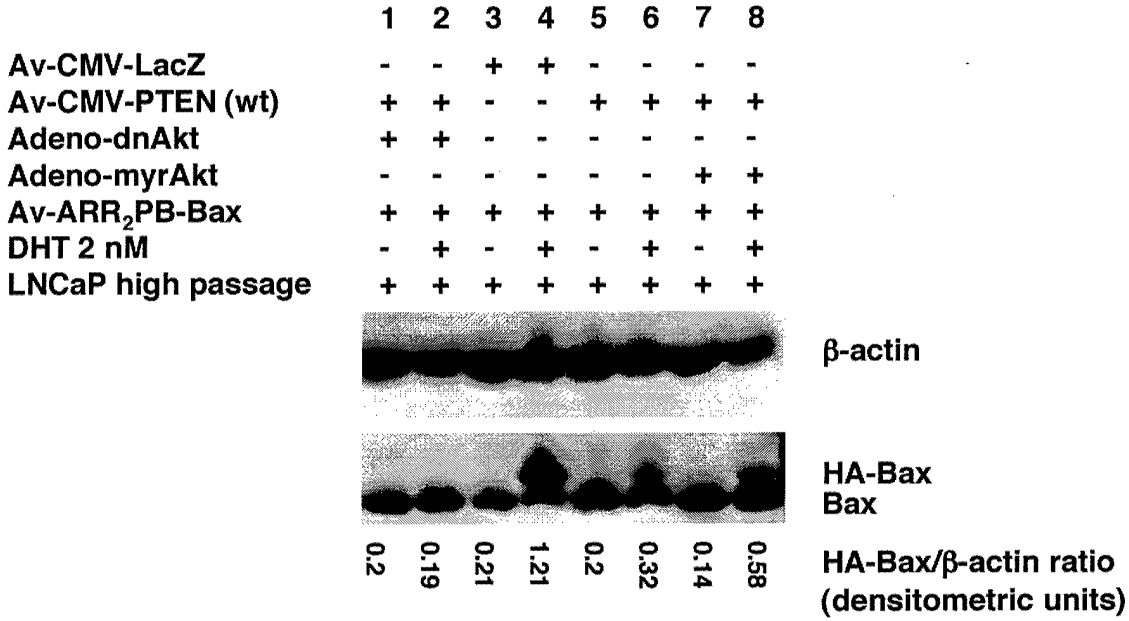

1 2 3 4 5 6 7 8
Av-CMV-LacZ - - + + - - - -
Av-CMV-PTEN (wt) + + - - + + + +
Adeno-dnAkt + + - - - - - -
Adeno-myrAkt - - - - - - + +
Av-ARR2PB-Bax + + + + + + + +
DHT 2 nM - + - + - + - +
LNCaP high passage + + + + + + + +
β-actin
HA-Bax
Bax
0.2 0.19 0.21 1.21 0.2 0.32 0.14 0.58
HA-Bax/β-actin ratio
(densitometric units)

Figure 4

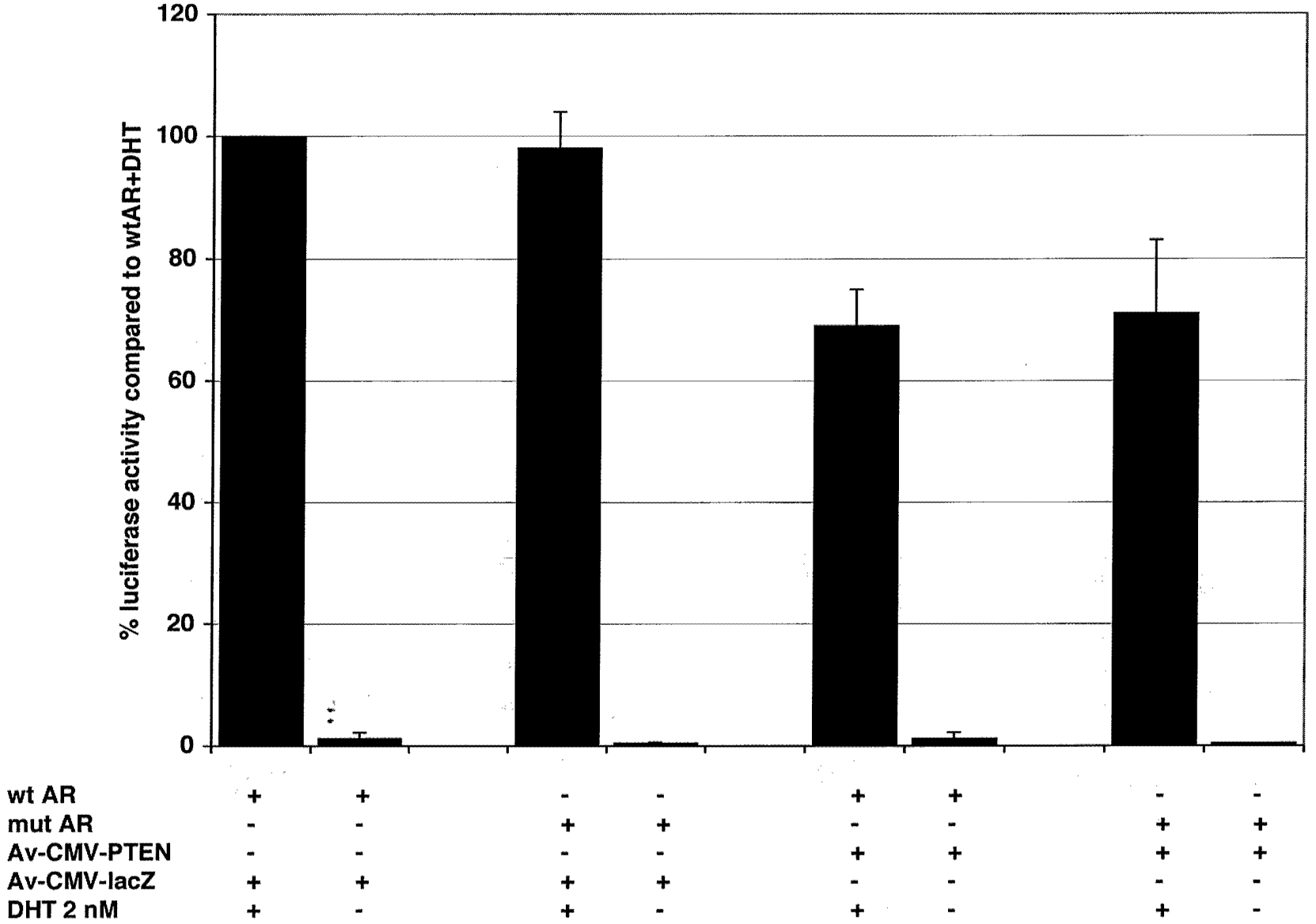

120
100
80
60
40
20
0
% luciferase activity compared to wtAR+DHT
wt AR + + - - + + - -
mut AR - - + + - - + +
Av-CMV-PTEN - - - - + + + +
Av-CMV-lacZ + + + + - - - -
DHT 2 nM + - + - + - + -

Figure 5